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The Journal of Biochemistry, Vol. 46, No. 4, 1959

# THE MODE OF ACTION OF TRYPSIN UPON SYNTHETIC POLY-ε-AMINOCAPROYL-α-ALANINES*

By MITUO EBATA

*(From the Department of Chemistry, Faculty of Science, Kanazawa University, Kanazawa)*

(Received for publication, December 20, 1957)

In previous papers (*1*, *2*) we reported that the synthetic linear polymers, poly-ε-aminocaproyl-α-alanines, can be partly hydrolysed by the action of trypsin if relatively high concentrations of the enzyme are employed. This finding is important in connection to the problem of substrate specificity of trypsin.

The present study was undertaken to identify the products of tryptic hydrolysis of poly-ε-aminocaproyl-α-alanines and to examine the behavior of trypsin upon several synthetic substrates for the aim of finding a clue to the substrate specificity of the enzyme.

## EXPERIMENTAL

Poly-ε-aminocaproyl-DL-alanine, poly-ε-aminocaproyl-L-alanine and the trypsin preparations have been described previously (*2*). Dialysable cleavage products from the polymer were isolated as follows: To 100 ml. of dialysed 0.2 per cent solution of poly-ε-aminocaproyl-DL-alanine (pH 7.1), 5.0 mg. of Trypsin II was added, and 50 ml. of the mixture was incubated at 25° and the other 50 ml. was also incubated at 55° for 60 minutes. After incubation the hydrolysis was stopped by addition of a large amount of alcohol. Then the mixtures were dialysed against 1,000 ml. of distilled water for 24 hours in an ice box. Dialysed substances of cleavage products were concentrated up below 40° and dried *in vacuo*. The products of small molecular weight thus obtained were investigated by paper chromatographic analysis. The free amino acid or peptides were extracted from the chromatogram with water and determined quantitatively by the ninhydrin method described below. The DNP-amino acids were identified and estimated by quantitative paper chromatographic analysis using a solvent mixture, phenol-isoamylalcohol-water (1 : 1 : 1) (*4*, *5*).

The rate of cleavage of the peptides and acid amides was followed by measurement of the formation of free amino groups or ammonia by ninhydrin method of Moore and Stein (*6*). In several experiments with acid amides the Conway's microdiffusion method was employed. The rate of disappearance of acid esters and peptide esters was followed by the hydroxamic acid method of Hestrin (*7*). In all cases, control experiments were made to determine the lability of the substrate, in the absence of the enzyme to be added, at the same pH and temperature. Where no experimental data are given for such control experiments the substrates were found to be stable in the course of the experiments.

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* A part of this work was presented at the Symposium of Protein Structure in Tokyo, Nomvember, 1955, the 9th Annual Meeting of the Chemical Society of Japan in Kyoto, April, 1956 and the 29th Annual Meeting of the Japanese Biochemical Society in Fukuoka, November, 1956.

*ε-Aminocaproic Acid Methyl Ester Hydrochloride*—This compound was prepared by the treatment of ε-aminocaproic acid hydrochloride with HCl in absolute methanol. Yield, 99 per cent; m.p., 79°.

*Analysis* Calcd. for $C_7H_{16}O_2NCl$: N, 7.72 per cent
Found: N, 7.68

*ε-Aminocaproic Acid Ethyl Ester Hydrochloride*—This substance was prepared by the treatment of ε-aminocaproic acid hydrochloride with HCl in absolute ethanol. Yield, 99 per cent; m.p., 58°.

*ε-Benzoylaminocaproic Acid Methyl Ester*—ε-Aminocaproic acid was benzoylated in the usual manner; m.p. 77–80°. The esterification reaction gave the desired compound in 99 per cent yield; oily product.

*Analysis* Calcd. for $C_{14}H_{19}O_3N$: N, 5.63 per cent
Found: N, 5.40

*ε-Aminocaproamide Hydrochloride*—ε-Carbobenzoxyaminocaproamide was obtained as a product melting at 123–124°; yield, 78 per cent.

*Analysis* Calcd. for $C_{14}H_{20}O_3N_2$: N, 10.58 per cent
Found: N, 10.17

The amide was converted to the ε-aminocaproamide hydrochloride in the usual hydrogenolysis manner. Yield, 96 per cent; m.p., 144°.

*Analysis* Calcd. for $C_6H_{15}ON_2Cl$: N, 15.33 per cent
Found: N, 15.32

*ε-Benzoylaminocaproamide*—ε-Benzoylaminocaproic acid was treated with thionylchloride in chloroform and converted to the amide (over-all yield, 80 per cent); m.p., 138–139°.

*Analysis* Calcd. for $C_{13}H_{18}O_2N_2$: N, 11.95 per cent
Found: N, 11.88

*ε-Aminocaproyl-L-alanine*—The carbobenzoxy depeptide was obtained by the usual coupling reaction in a crystalline form; over-all yield 87 per cent.

*Analysis* Calcd. for $C_{17}H_{24}O_5N_2$: N, 8.33 per cent
Found: N, 8.27

Hydrogenolysis gave the dipeptide in a yield of 81 per cent; $[\alpha]^{20}_{D}-23.0°$ (water: c 2.0 per cent).

*Analysis* Calcd. for $C_9N_{18}O_3N_2$: N, 13.83 per cent
Found: N, 13.44

*ε-Aminocaproyl-D-alanine*—The carbobenzoxy peptide was obtained as a product melting at 116°; yield, 69 per cent.

*Analysis* Calcd. for $C_{17}H_{24}O_5N_2$: N, 8.33 per cent
Found: N, 8.38

Hydrogenolysis gave the disired dipeptide in a yield of 80 per cent; m.p., 155°; $[\alpha]^{20}_{D}+23.0°$ (water; c, 2.0 per cent).

*Analysis* Calcd. for $C_9H_{18}O_3N_2$: N, 13.83 per cent
Found: N, 13.42

*ε-Benzoylaminocaproyl-L-alanine*—The coupling product was obtained in 62 per cent yield; $[\alpha]^{20}_{D}-24.5°$ (50 per cent alcohol; c, 2.0 per cent).

*Analysis* Calcd. for $C_{16}H_{22}O_4N_2$: N, 9.14 per cent
Found: N, 9.06

*ε-Benzoylaminocaproyl-*D*-alanine*—This substance was obtained in crystalline form; yield, 71 per cent; m.p., 102°; $[\alpha]^{20}_{D}+24.5°$ (50 per cent alcohol; c, 2.0 per cent).

*Analysis* Calcd. for $C_{16}H_{22}O_4N_2$: N, 9.14 per cent
Found: N, 9.23

*ε-Carbonbenzoxyaminocaproyl-*L*-alanine Methyl Ester*—This compound was prepared from ε-carbobenzoxyaminocaproylchloride and L-alanine methyl ester in a ether solution. Yield, 87 per cent; m.p., 67°.

*Analysis* Calcd. for $C_{18}H_{26}O_5N_2$: N, 7.99 per cent
Found: N, 8.03

*ε-Aminocaproyl-*L*-alanine Methyl Ester Hydrochloride*—Hydrogenolysis of the carbobenzoxy compound gave a non-crystalline solid in 83 per cent yield; m.p., 57°; $[\alpha]^{25}_{D}-20.4°$ (water; c, 2.0 per cent).

*Analysis* Calcd. for $C_{10}H_{21}O_3N_2Cl$: N, 11.08 per cent
Found: N, 11.02

*ε-Carbobenzoxyaminocaproyl-*L*-alaninamide*—ε-Carbobenzoxyaminocaproyl-L-alanine methyl ester was converted to the amide. Yield, 90 per cent; m.p., 142°; $[\alpha]^{20}_{D}-14.7°$ (methanol; c, 2.0 per cent).

*Analysis* Calcd. for $C_{17}H_{25}O_4N_3$: N, 12.53 per cent
Found: N, 12.12

*ε-Aminocaproyl-*L*-alaninamide*—Hydrogenolysis of the carbobenzoxy compound gave the product in 98 per cent yield. The substance was recrystallized from methanol-ether; m.p., 122°; $[\alpha]^{25}_{D}-28.2°$ (methanol; c, 1.5 per cent).

*Analysis* Calcd. for $C_9H_{19}O_2N_3$: N, 20.88 per cent
Found: N, 20.38

L*-Alanyl-ε-aminocaproic Acid*—The coupling reaction gave the oily carbobenzoxydipeptide, yield, 87 per cent.

*Analysis* Calcd. for $C_{17}H_{24}O_5N_2$: N, 8.33 per cent
Found: N, 8.34

Hydrogenolysis gave the dipeptide in a yield of 81 per cent; $[\alpha]^{20}_{D}-31.2°$ (30 per cent alcohol; c, 2.0 per cent).

*Analysis* Calcd. for $C_9H_{18}O_3N_2$: N, 13.83 per cent
Found: N, 13.44

D*-Alanyl-ε-aminocaproic Acid*—The coupling reaction gave a desired carbobenzoxydipeptide in a yield of 69 per cent.

*Analysis* Calcd. for $C_{17}H_{24}O_5N_2$: N, 8.33 per cent
Found: N, 8.33

Hydrogenolysis of the carbobenzoxy compound gave non-crystalline solid in 80 per cent yield; $[\alpha]^{20}_{D}+31.2°$ (30 per cent methanol; c, 2.0 per cent).

*Analysis* Calcd. for $C_9H_{18}O_3N_2$: N, 13.83 per cent
Found: N, 13.42

## RESULTS

*Products of Tryptic Hydrolysis of Poly-ε-aminocaproyl-α-alanines*—A search for the cleavage products of poly-ε-aminocaproyl-DL-alanine and poyl-ε-aminocaproyl-L-alanine by paper partition chromatography revealed that small amounts of free alanine and ε-aminocaproic acid, together with a large amount of dialysable fragments, were released from the polymers. These dialysable peptides gave alanine and ε-aminocaproic acid on complete hydrolysis with 6 *N* hydrochloric acid (Fig. 1). Sometimes traces of free ε-aminocaproyl-L-alanine were observed as a product especially when ploy-ε-aminocaproyl-L-alanine was employed as the substrate. Qualitatively identical results were obtained at different temperatures indicating that the mode of cleavage was unaffected by the heat coagulable property (*2*). No spot was observed when control samples without either substrate or enzyme were analysed.

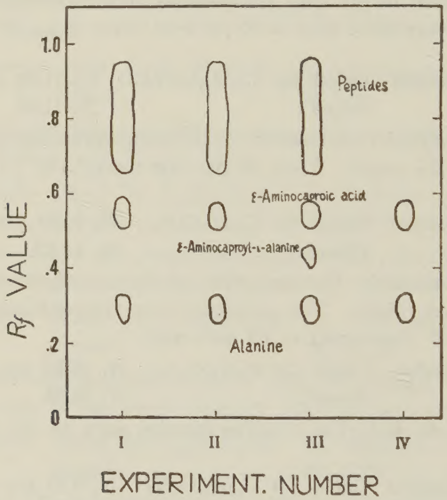


FIG. 1. A Paper chromatogram of the hydrolytic products of poly-ε-aminocaproyl-α-alanines.

I: Poly-ε-aminocaproyl-DL-alanine; temperature of tryptic attack, 25°.

II: Poly-ε-aminocaproyl-DL-alanine; temperature, 55°.

III: Poly-ε-aminocaproyl-L-alanine; temperature, 25°.

IV: Complete hydrolytic products of peptides formed in experment I with 6 *N* hydrochloric acid for 4 hours.

Solvent mixture: *n*-butanol, acetic acid and water (4 : 1 : 1), filter paper: Toyo Roshi No. 50.

Quantitative experiments as can be seen from Table I, showed that about half mole of ε-aminocaproic acid was released from one molecule of the poly-ε-aminocaproyl-DL-alanine. A considerable amount of free alanine, though smaller than that of ε-aminocaproic acid, was also observed. Poly-ε-aminocaproyl-L-alanine, on the other hand, gave very small amounts of the free amino acids and peptides, due to making relative resistance of the L-alanine polymer to trypsin. Such a resistance may be partly caused by the smaller molecular weight of the L-polymer (L-alanine polymer 7,000;

DL-alanine polymer 21,800). No products of hydrolysis could be detected when $\alpha$-chymotrypsin or carboxypeptidase was used instead of trypsin.

The dialysable peptides were hydrolysed with 6 *N* hydrochloric acid and the mean grade of polymerization was estimated to be 6 from their

TABLE I

*Investigation of Hydrolytic Products of Poly-ε-aminocaproyl-α-alanines by the Action of Trypsin*

| Polymer | Enzyme | Conditions | Formation of | | | N-terminal residues | | |
|---|---|---|---|---|---|---|---|---|
| | | | ε-Amino caproic acid ($\mu$M) | Alanine ($\mu$M) | Peptides ($\mu$M) | Calculated from mol. wt. of the polymer ($\mu$M) | Found (DNP method) ε-Amino-caproic acid ($\mu$M) | Found (DNP method) Alanine ($\mu$M) |
| Poly-ε-amino-caproyl-DL-alanine 90 *mg.* | Trypsin II, 2.0 *mg.* | 35° 2 *hrs.* | 1.90 | 1.35 | 7.89 | 4.13 | — | — |
| „ 80 *mg.* | „ „ | „ „ | 1.95 | 1.02 | 2.91 | 3.70 | — | — |
| „ 50 *mg.* | Trypsin I, 5.5 *mg.* | 30° 1 *hr.* | 1.55 | 1.16 | 2.85 | 2.30 | — | — |
| „ 110 *mg.* | — | — | — | — | — | 12.40 | 9.0 | 0.4 |
| Poly-ε-amino-caproyl-L-alanine 70 *mg.* | Trypsin II, 1.8 *mg.* | 37° 3 *hrs.* | 0.51 | 0.40 | 0.40 | 10.0 | — | — |
| „ 87 *mg.* | — | — | — | — | — | 5.04 | 4.5 | 0.1 |

TABLE II

*Investigation of the Released Peptides from the Polymers*

| Polymer | Enzyme | Conditions | Peptides formed ($\mu$M) | Free amino group after hydrolysis with 6 *N* HCl ($\mu$M) | Mean degree of polymerization | N-Terminal residues of peptides | |
|---|---|---|---|---|---|---|---|
| | | | | | | ε-Amino-caproic acid ($\mu$M) | Alanine ($\mu$M) |
| Poly-ε-amino-caproyl-DL-alanine 80 *mg.* | Trypsin II, 2.0 *mg.* | 35°, 2 *hrs.* | 2.91 | 35.4 | 6.1 | — | — |
| „ 50 *mg.* | Trypsin I, 5.5 *mg.* | 30°, 1 *hr.* | 2.85 | 37.2 | 6.5 | | |
| „ 248 *mg.* | Trypsin II, 4.0 *mg.* | 35°, 3 *hrs.* | 8.2 | — | — | 0.8 | 5.8 |
| Poly-$\alpha$-amino-caproyl-L-alanine 70 *mg.* | Trypsin II, 1.8 *mg.* | 37°, 3 *hrs.* | 0.40 | 2.7 | 6.7 | — | — |

TABLE III

*Action of Trypsin upon ε-Aminocaproic Acid Derivatives*

| Substrate | Concentration | | pH | Analitical method* | Hydrolysis (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Enzyme (Protein N. μg./ml.) | Substrate (*M*) | | | 10 | 20 | 30 | 40 | 60 | 90 | 120 | 180 (*min.*) |
| ε-Aminocaproyl-L-alanine | 9.7 | 0.05 | 7.8 | N | | | | | | | 0.3 | 0.6 |
| | 7.5 | 0.005 | ” | ” | 0.2 | 0.7 | | 0.8 | 0.9 | 1.2 | 1.3 | |
| | 9.7 | 0.001 | ” | ” | | 0.6 | | 0.8 | 1.2 | 1.4 | 1.6 | |
| ε-Aminocaproyl-D-alanine | 9.7 | 0.05 | 7.8 | N | | | 0.0 | | 0.0 | | 0.0 | 0.0 |
| ε-Aminocaproamide | 9.7 | 0.05 | 7.8 | N | | | | | | | 0.7 | |
| | ” | ” | ” | C | | 1.9 | | 2.6 | 2.9 | | | |
| | ” | 0.001 | ” | N | | | | | 1.2 | | 2.4 | 3.7 |
| ε-Aminocaproic acid methyl ester | 15.0 | 0.05 | 7.8 | H | 1.2 | 6.5 | 16 | | 16 | | 28 | |
| | ” | 0.005 | ” | ” | 31 | 51 | 83 | | 100 | | | |
| | 9.7 | 0.05 | 9.2 | ” | | | | | 14 | | 26 | 44 |
| | ” | 0.005 | ” | H | 36 | 50 | | | | | | |
| ε-Aminocaproic acid ethyl ester | 9.7 | 0.05 | 9.2 | H | 2 | 5 | 12 | | 15 | 22 | 28 | 39 |
| ε-Aminocaproyl-L-alaninamide | 9.7 | 0.05 | 6.6 | C | | | | | 0.2 | 0.3 | 0.4 | 0.4 |
| | 9.7 | ” | 7.1 | ” | 0.0 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| ε-Aminocaproyl-L-alanine methyl ester | 9.7 | 0.05 | 6.6 | H | 0.8 | | | | | 1.5 | 1.8 | 2.3 |
| | 9.1 | 0.025 | 7.8 | N | | 0.2 | | 0.4 | 0.5 | 0.6 | | |
| L-Alanyl-ε-aminocaproic acid | 9.7 | 0.05 | 7.8 | N | | | 0.0 | | 0.0 | 0.0 | 0.0 | 0.0 |
| D-Alanyl-ε-aminocaproic acid | 9.7 | 0.05 | 7.8 | N | | | 0.0 | | 0.0 | 0.0 | 0.0 | 0.0 |

* N, ninhydrin method; C, Conway's microdiffusion method; H, hydroxamic acid method.
In all cases Trypsin II preparation was used.

## TABLE IV

*Action of Tyrpsin upon Benzoyl and Carbobenzoxyaminocaproic Acid Derivatives*

| Substrate | Concentration | | pH | Analytical method* | Hydrolysis (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Enzyme (Protein N. $\mu g./ml.$) | Substrate ($M$) | | | 10 | 20 | 30 | 40 | 60 | 90 | 120 | 180 (*min.*) |
| ε-Benzoylaminocaproyl-L-alanine⁺ | 9.7 | 0.05 | 7.8 | N | | | | | 0.01 | | 0.03 | 0.03 |
| ε-Benzoylaminocaproyl-D-alanine⁺ | 9.7 | 0.05 | 7.8 | N | | | 0.00 | | 0.00 | | 0.00 | 0.01 |
| ε-Benzoylaminocaproamide | 19.4 | 0.05 | 7.8 | C | | 0.0 | | 0.0 | 0.0 | | | |
| ε-Carbobenzoxyaminocaproamide⁺ | 19.4 | 0.005 | 7.8 | N | | | 0.00 | | 0.00 | | 0.0 | |
| ε-Benzoylaminocaproic acid methyl ester⁺ | 19.4 | 0.005 | 7.8 | H | 0.0 | | 0.0 | | 0.0 | | | |
| ε-Carbobenzoxyaminocaproyl-L-alanine-amide⁺ | 9.7 | 0.05 | 6.6 | N | 0.0 | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ε-Carbobenzoxyaminocaproyl-L-alanine methyl ester⁺ | 9.7 | 0.05 | 6.6 | H | 0.0 | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Carbobenzoxy-L-alanyl-ε-aminocaproic acid⁺ | 9.7 | 0.05 | 7.8 | N | | | 0.00 | | 0.00 | | | 0.00 |
| Carbobenzoxy-D-alanyl-ε-aminocaproic acid⁺ | 9.7 | 0.05 | 7.8 | N | | | 0.00 | | 0.00 | | | 0.00 |

* Analtical method was described as Table III..

+ Tested in the presence of 30 per cent methanol.

The enzyme used was Trypsin II in all cases.

ninhydrin colour values, *i.e.*, one peptide consisted of six ε-aminocaproic acid and six alanine residues on average. Alanine was found as the N-terminal amino acid residue of the peptide by the dinitrophenylation method of Sanger as shown in Table II.

*Behavior of Trypsin upon ε-Aminocaproic Acid Derivatives*—The behavior of trypsin upon a number of ε-amino-*n*-caproyl compounds was investigated. Tables III and IV show the results obtained with ε-aminocaprolyl, ε-benzoyl- and ε-carbobenzoxyaminocaproyl derivatives as substrates.

Both ε-aminocaproyl-L-alanine and ε-aminocaproamide were found to be hydrolysed to small extents by trypsin. Much more extensive hydrolysis was observed with methyl and ethyl esters of ε-aminocaproic acid at pH 7.8–9.2 as shown in Table III. Amide and ester of ε-aminocaproyl-L-alanine were slowly hydrolysed under the same conditions, liberating a small amount of the free dipeptide or ε-aminocaproic acid, as demonstrated by paper chromatography using *n*-buthanol-acetic acid-water (4 : 1 : 1) as the solvent. L-Alanyl-ε-aminocaproic acid and all of the D-alanine-containing peptides tested were completely resistant to the enzyme action as can be seen from Table III.

It will be seen from Table IV that all these compounds were completely resistant to the action of trypsin if their free ε-amino groups were blocked with benzoyl or carbobenzoxy groups. Apparently, the presence of free ε-amino groups in the substrate makes the peptide, amide or ester bonds sensitive to the attack by trypsin. Since the compounds described in Table IV were hardly soluble in water, their susceptibility to the enzyme was tested in 30 per cent methanol solution. It seems that the enzymatic reaction was not inhibited by methanol in this case, since no inhibition was caused by methanol in the case of the compounds described in Table III.

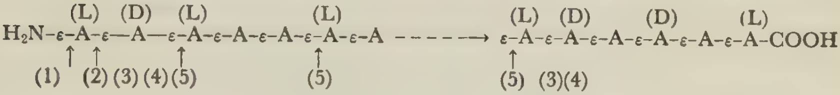
## DISCUSSION

The studies described in this communication, together with the results of the previous investigations on poly-ε-aminocaproyl-α-alanines (*1*, *2*) clearly indicate that trypsin is able to hydrolyse certain peptide bonds of poly-ε-aminocaproyl-L-alanine. The bond of N-terminal ε-aminocaproic acid residue in the polymer seems to be especially susceptible. However, the results described above appear to suggest a possible mode of action of trypsin on the polymer as illustrated in Diagram 1.

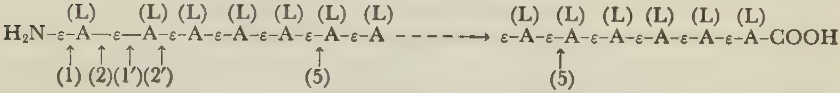
Tryptic cleavage of the peptide bond adjacent to the N-terminal amino acid residue (position 1), was suggested from the fact that the peptide, amide or ester of the ε-aminocaproic acid are considerably susceptible to the enzyme action (Table III). But if the second alanine residue was D-form in the poly-ε-aminocaproyl-DL-alanine the position would not be hydrolysed since ε-aminocaproyl-D-alanine was not hydrolysed by the tryptic action. The possible scheme described above also shows that free ε-aminocaproyl-L-alanine will be released from the polymer if the position 2 is attacked, and that the released compound will be again subjected to the enzyme action forming both free ε-aminocaproic acid and alanine. Further, the attack by trypsin at the position 1′ and 2′ is also suggested in the case of poly-ε-aminocaproyl-L-alanine.

DIAGRAM 1.

Suggested mode of action of trypsin upon poly-ε-aminocaproyl-α-alanines.


(L) (D) (L) (L) (L) (D) (D) (L)
H₂N-ε-A-ε—A—ε-A-ε-A-ε-A-ε-A-ε-A ------→ ε-A-ε-A-ε-A-ε-A-ε-A-ε-A-COOH
(1) (2) (3) (4) (5) (5) (5) (3)(4)

Poly-ε-aminocaproyl-DL-alanine


(L) (L) (L) (L) (L) (L) (L) (L) (L) (L) (L) (L) (L)
H₂N-ε-A—ε—A-ε-A-ε-A-ε-A-ε-A-ε-A ------→ ε-A-ε-A-ε-A-ε-A-ε-A-ε-A-COOH
(1) (2)(1′)(2′) (5) (5)

Poly-ε-aminocaproyl-L-alanine

ε: ε-aminocaproyl, $-NH \cdot (CH_2)_5 \cdot CO-$

A: alanyl, $-NH \cdot \underset{CH_3}{\underset{|}{CH}} \cdot CO-$

It was shown in this communication that dialysable peptides consisting of ε-aminocaproic acid and alanine are released from the polymer by the action of trypsin. This fact is quite unexplicable from the results obtained with synthetic simple ε-aminocaproic acid derivatives (Tables III and IV) and from the other available information on the specificity of the enzyme. The results reported here, however, clearly indicate that these peptides are released from the polymer by the action of trypsin and not by the other proteolytic enzymes such as α-chymotrypsin and carboxypeptidase contained in the trypsin preparations as impurities. A possible explanation may be that trypsin may exert somewhat different behaviors to high molecular compounds as compared to low molecular substrates. Thus, it seems probable that the inner peptide bonds on the polymer, position 5, can also be hydrolysed by the action of trypsin, though position 3 are not hydrolysed. It is further conceivable that the unusual hydrolysis was induced by some protein-like properties of the polymer. In fact, it has been observed that the higher is the molecular weight of the polymer, the more easily it is hydrolysed.

## SUMMARY

Hydrolysis products of synthetic linear polymers, both poly-ε-aminocaproyl-DL-alanine and poly-ε-aminocaproyl-L-alanine by the action of trypsin were investigated by paper chromatographic analysis. Small amounts of free alanine and ε-aminocaproic acid together with large amount of dialysable peptides were found as the dialysable hydrolysis products. Sometimes traces of free ε-aminocaproyl-L-alanine could be observed in the case of poly-ε-aminocaproyl-L-alanine. Furthermore, some studies on the substrate

specificity of the enzyme were carried out using a number of synthetic substrates related to the polymer, and a possible mode of action of trypsin upon the poly-ε-aminocaproyl-α-alanines was discussed.

The author wished to express his hearty thanks to Prof. F. Egami of Nagoya University, Prof. J. Noguchi of this university, Dr. R. Sato, Osaka University and to Dr. N. Sakota, Dainippon Zoki Laboratory, for their kind encouragements throughout the course of this work. The author is also grateful to Mr. K. Morita for his technical assistances.

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# HYDROLYSIS OF ε-AMINOCAPROYL COMPOUNDS BY TRYPSIN*

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It has been known that trypsin, an important proteolytic enzyme, can hydrolysis only lysyl and arginyl peptide, amide, and ester linkages (*1*, *2*). Recently it has been shown that benzoyl-L-histidine ester (*3*) and *n*-fatty acid esters (*4*) are also susceptible to the action of the enzyme. Shapiro and Doherty (*5*) have briefly reported that ε-aminocaproic acid esters can also be hydrolysed by trypsin. The author has independently found in the course of a study on tryptic hydrolysis of poly-ε-aminocaproyl-α-alanines (*6*) that the ε-aminocaproyl peptides, amides and esters are hydrolysed by the action of trypsin.

The present paper deals with detailed studies concerning the hydrolysis of the ε-aminocaproyl compounds by the action of trypsin.

## EXPERIMENTAL

**Trypsin II** preparation (70 per cent purity of Trypsin I) described previously (*6*) was used throughout this study except for some special experiments conducted using Trypsin I. The rate of hydrolysis of peptides, amides and esters were determined as described previously (*12*). Control experiments were also performed in all cases. In several experiments using ornithine, δ-aminvaleric acid and γ-aminobutyric acid derivatives the electric alcohol-alkali titration method, a modification of the method of Willstäter *et al.* (*13*), was also employed.

The preparation of derivatives of ε-amincaproic acid, such as ε-aminocaproyl-L-alanine, ε-aminocaproamide and ε-aminocaproic acid methyl ester were already described previously (*12*).

*ε-Aminocaproyl-ε-aminocaproic Acid*—ε-Carbobenzoxyaminocaproyl-ε-aminocaproic acid was obtained in the usual manner from ε-carbobenzoxyaminocaproylchloride and ε-aminocaproic acid. Yield, 66 per cent; m.p., 88°.

*Analysis.* Calcd. for $C_{20}H_{30}O_5N_2$: N, 7.40 per cent
Found: N, 7.35

Hydrogenolysis of the carbobenzoxy compound gave the product; yield, 88 per cent; m.p., 177°.

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*Analysis.* Calcd. for $C_{12}H_{24}O_3N_2$: N, 11.46 per cent.
Found: N, 11.52

*Poly-ε-aminocaproic Acid (Polycaproamide)*—Polymerization of the ε-carbothiophenylaminocaproic acid (*14*) for 23 hours at 130° and then 3 hours at 160° gave this compound. Yield, 98 per cent; molecular weight, 2,000 (N-terminal amino group titration); mean degree of polymerization, about 18.

*Analysis* Calcd. for $H_2O(C_6H_{11}ON)_n$: N, 12.38 per cent ($n=\infty$)
Found: N, 12.27

This polymer was dissolved in a small amount of formic acid. The solution was added to a large amount of water, and the mixture dialysed against water for several days. The amorphous precipitate was used without drying.

*ε-Caprolactam*—The preparation manufactured by the Toyo Rayon Co. Ltd. was recrystallized from ether; m.p., 68.5°.

*Adipamide*—Adipic acid was treated with a large amount of thionyl chloride and converted to the amide (over-all yield, 50 per cent); m.p., 222°.

*Analysis* Calcd. for $C_6H_{12}O_2N_2$: N, 19.42 per cent
Found: N, 19.25

*δ-Aminovaleramide Hydrochloride*—The carbobenzoxy compound was obtained in crystalline form (yield, 20 per cent); m.p., 155°.

*Analysis* Calcd. for $C_{13}H_{18}O_3N_2$: N, 11.18 per cent
Found: N, 11.28

The corresponding amide was obtained as hydrochloride in a yield of 83 per cent; m.p., 147°.

*Analysis* Calcd. for $C_5H_{13}ON_2Cl$: N, 18.36 per cent
Found: N, 18.76

*δ-Aminovaleric Acid Methyl Ester Hydrochloride*—This substance was prepared by the treatment of δ-aminovaleric acid hydrochloride with HCl in absolute methanol; yield, 55 per cent; m.p., 138°.

*Analysis* Calcd. for $C_6H_{14}O_2NCl$: N, 8.36 per cent
Found: N, 8.47

DL-*Ornithine Ethyl Ester Dihydrochloride*—This substance was prepared by the treatment of DL-ornithine monohydrochloride with HCl in absolute ethanol. Yield, 95 per cent; m.p., 143–144°.

*Analysis* Calcd. for $C_7H_{18}O_2N_2Cl_2$: N 12.02, Cl 30.5 per cent
Found: N 12.14, Cl 29.8

*γ-Aminobutyric Acid Methyl Ester Hydrochloride*—γ-Aminobutyric acid hydrochloride was treated with HCl in absolute methanol, and was recystallized from alcohol-ether; yield, 91 per cent; m.p., 119°.

*Analysis* Calcd. for $C_5H_{12}O_2NCl$: N, 9.13 per cent
Found: N, 9.12

*β-Alanine Methyl Ester Hydrochloride*—β-Brompropionic acid was converted to β-alanine and esterified with absolute methanol; yield, 50 per cent; m.p., 91°.

*Analysis* Calcd. for $C_4H_{10}O_2NCl$: N, 10.02 per cent
Found: N, 9.93

*β-Alaninamide Hydrochloride*—β-alanine methyl ester hydrochloride was converted to the amide; yield, 60 per cent.

*Analysis* Calcd. for $C_3H_9ON_2Cl$: N, 22.48 per cent
Found: N, 18.30

## RESULTS

In Table I are presented the data indicating that various ε-aminocaproyl compounds can be hydrolysed by the action of trypsin. Among the compounds tested the methyl ester of ε-aminocaproic acid was by far most susceptible to the tryptic attack. ε-Aminocaproyl-L-alanine methyl ester seems to suffer hydrolysis at two positions, as evidenced by paper chromatographic detection of both ε-aminocaproic acid and alanine in the product (*6*). ε-Aminocaproyl-ε-aminocaproic acid was also attacked by the enzyme as shown in the table. It is interesting that poly-ε-aminocaproic acid (polycaproamide) was also slowly hydrolysed in an amorphous state though the polymer was almost insoluble in water. Free ε-aminocaproic acid might be released from the N-terminal part of the polymer in succession.

### TABLE I

*Action of Trypsin on ε-Aminocaproyl Compounds*

| Substrate | Substrate concentration (*M*) | pH | Analytical method* | Hydrolysis (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 20 | 40 | 60 | 90 | 120 | 180 (*min.*) |
| ε-Aminocaproic acid methyl ester | 0.05 | 9.2 | H | | | 14 | | 26 | 44 |
| ε-Aminocaproamide | 0.05 | 7.8 | C | 1.9 | 2.6 | 3 | | | |
| ε-Aminocaproyl-L-alanine | 0.05 | 7.8 | N | | | | | 0.3 | 0.6 |
| ε-Aminocaproyl-L-alanine methyl ester | 0.025 | 7.8 | N | 0.2 | 0.4 | 0.5 | 0.6 | | |
| | 0.05 | 6.6 | H | 0.8 | | | 1.5 | 1.8 | 2.3 |
| ε-Aminocaproyl-ε-aminocaproic acid | 0.005 | 6.4 | N | 0.6 | 1.4 | 2.2 | | | |
| Poly-ε-aminocaproic acid** (amorphous state) | 0.0015 | 7.1 | N | 0.05 | 0.08 | 0.13 | 0.19 | 0.21 | 0.26 |

* Analytical method: H, hydroxamic acid method; C, Conway's microdiffusion method; N, ninhydrin method.

** A precipitate was present.

All reaction mixtures contained 9.7 μg./ml. (protein N) of Trypsin II.

Effect of pH on the hydrolysis of ε-aminocaproyl compounds by the enzyme action was investigated as shown in Fig. 1. The optimum pH's were found to be pH 9.0–9.2 (for ε-aminocaproic acid methyl ester), pH 6.2–6.3 (for ε-aminocaproamide), pH 7.0–7.2 (for ε-aminocaproyl-L-alanine) and pH 6.7–6.8 (for ε-aminocaproyl-L-alanine methyl ester).

The affinity of the enzyme to several ε-aminocaproyl compounds was determined at each optimum pH by the Lineweaver-Burk graphical

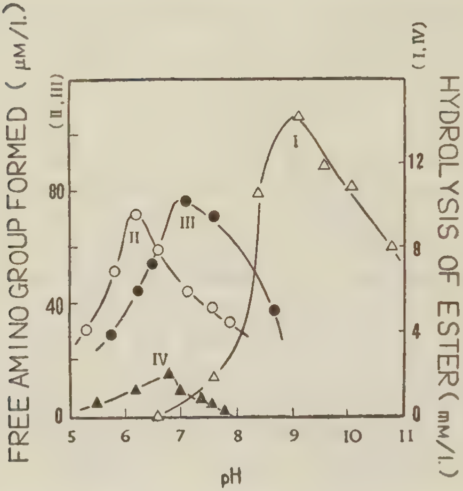

FIG. 1. Effect of pH on the hydrolysis of ε-aminocaproyl compounds.

I: ε-Aminocaproic acid methyl ester, 0.05 *M*; time, 60 minutes.

II: ε-Aminocaproamide, 0.0025 *M*; time, 120 minutes.

III: ε-Aminocaproyl-L-alanine, 0.005 *M*; time 90 minutes.

IV: ε-Aminocaproyl-L-alanine methyl ester, 0.04 *M*; time, 60 minutes.

Buffers; pH 5–8, phosphate buffer; pH 8–9, phosphate-borate buffer; pH 9–11, borax buffer.

Each reaction contained 9.7 μg./ml. (protein N) of Trypsin II, and incubation was carried out at 25°.

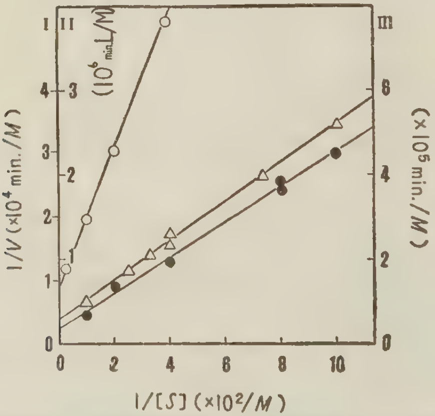

FIG. 2. Influence of concentration of ε-aminocaproyl compounds on the rate of reaction.

I: ε-Aminocaproic acid methyl ester; pH 9.2; time, 10 minutes. (△)

II: ε-Aminocaproamide; pH 6.4; time, 30 minutes. (○)

III: ε-Aminocaproyl-L-alanine; pH 7.2; time, 60 minutes. (●)

Each reaction mixture contained 9.7 μg./ml. of Trypsin II, and incubation was carried out at 25°.

treatment (*7*) (Fig. 2). The Michaelis constant $K_m$ was $16.9\times10^{-3}\,M$ for ε-aminocaproic acid methyl ester, $10.4\times10^{-3}\,M$ for ε-aminocaproamide, $10\times10^{-3}\,M$ for ε-aminocaproyl-L-alanine and $7\times10^{-3}\,M$ for ε-aminocaproyl-L-alanine methyl ester.

It was found that the tryptic digestion of ε-aminocaproyl compounds follows the kinetics of the first order reaction and that the velocity constant is proportional to the trypsin concentration (Fig. 3, Table II). The proteolytic coefficient $k_3$ was estimated to be $145\times10^{-3}$ (with ε-aminocaproic acid methyl ester as substrate), $2\times10^{-3}$ (ε-aminocaproamide and ε-aminocaproyl-L-alanine) and $7\times10^{-3}$ (ε-aminocaproyl-L-alanine methyl ester) as shown in Table II.

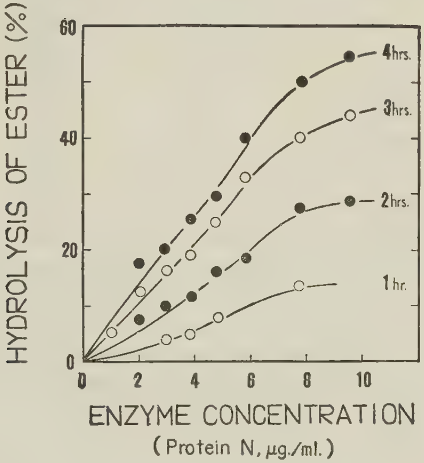

**FIG. 3.** Influence of the enzyme concentration of the hydrolysis of ε-aminocaproic acid methyl ester.

Each reaction mixture contained 0.05 *M* of the substrate, and 0.067 *M* borax buffer (pH 9.2) and each concentration of the Trypsin I. Incubation temperature, 25°

It is necessary to establish that these ε-aminocaproyl compounds are not hydrolysable by other proteolytic enzymes or esterases contained in the trypsin preparation as impurities. In order to exclude such possibilities several experiments were carried out. The rates of hydrolysis of ε-aminocaproic acid methyl ester by three different trypsin preparations were compared; the preparations used being Trypsin I (twice recrystallized) and two kinds of Trypsin II (63 per cent and 70 per cent purity). No difference was, however, detected among the rates observed in the three experiments if equal trypsin units were employed as shown in Fig. 4. This suggests that no enzyme other than trypsin itself capable of hydrolysing the ε-aminocaproyl compound was present in the partially purified preparation, Trypsin II. It has been known that trypsin is stable in an acid solution (pH 2) (*8*). In the present study, as is shown in Table III, it was found that the

TABLE II

*Proteolytic Coefficient of $\varepsilon$-Aminocaproyl Compounds*

| Substrate | Enzyme concentration (protein N $\mu g./ml.$) | Reaction velocity constant $K$ ($10^3$) | Proteolytic coefficient $k_3$ ($10^3$) | |
|---|---|---|---|---|
| $\varepsilon$-Aminocaproic acid methyl ester (pH 9.2) | 9.70 | 1.34 | 138 | 145 |
| | 7.76 | 1.14 | 147 | |
| | 5.82 | 0.89 | 153 | |
| | 4.85 | 0.77 | 159 | |
| | 3.88 | 0.51 | 132 | |
| | 2.91 | 0.42 | 144 | |
| $\varepsilon$-Aminocaproamide (pH 6.3) | 9.1 | 0.017 | 1.9 | 2 |
| | 7.7 | 0.013 | 1.6 | |
| | 5.6 | 0.012 | 2.4 | |
| $\varepsilon$-Aminocaproyl-L-alanine (pH 7.2) | 11.2 | 0.023 | 2.1 | 2 |
| | 9.1 | 0.020 | 2.2 | |
| | 7.7 | 0.013 | 1.7 | |
| $\varepsilon$-Aminocaproyl-L-alanine methyl ester | | | | |
| Hydrolysis of the peptide linkage (pH 7.8) | 9.1 | 0·024 | 2.6 | |
| Hydrolysis of the ester linkage (pH 6.6) | 9.7 | 0.070 | 7 | |

Each reaction mixture contained 0.05 $M$ the each substrate, 0.067 $M$ buffer which was described in Fig. 1 (pH, as described in the table) and each concentrations of Trypsin II preparation. Incubation was carried out for 10, 20, 30, 40, 60 and 120 minutes at 25°. The $k_3$ is expressed in moles/liter/min./mg. enzyme N/ml.

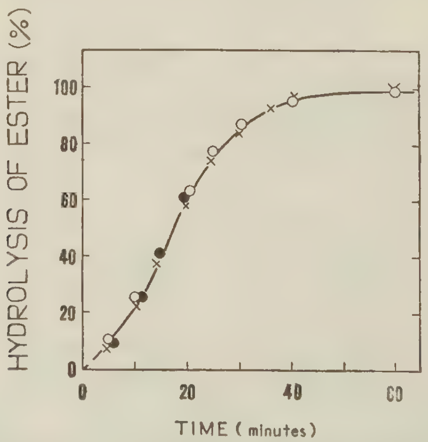

FIG. 4. Hydrolysis of $\varepsilon$-aminocaproic acid methyl ester by the action of several trypsin preparation.

Each reaction contained 0.005 $M$ the substrate, $2.6 \times 10^{-3}$ units/ml. of the enzyme and 0.1 $M$ borax buffer (pH 9.2).

Incubation temperature, 25°. × : Trypsin I (twice recystallized), ○· Trypsin II (purity, 63%), ●· Trypsin II (purity, 70%).

treatment of preparation with 0.01 *N* hydrochloric acid (pH 2.18) for 24 hours at 25° did not decrease the activity at all to hydrolyse ε-aminocaproic acid methyl ester. This fact also suggests that the enzyme which catalyzes this hydrolysis reaction is as stable to acid treatment as trypsin. Further, hydrolysis of ε-aminocaproic acid methyl ester was found to be strongly inhibited by small amount of diisopropylfluorophosphate or carbonyl reagents such as hydroxylamine, as in the case of normal tryptic catalysis. Taking all facts just described into consideration, it seems justified to assume that trypsin itself is in fact responsible for the hydrolysis of not only lysyl or arginyl compounds but also ε-aminocaproyl compounds.

TABLE III

*Acid Treatment of Enzyme and its Activity on the Hydrolysis of ε-Aminocaproic Acid Methyl Ester*

| Time (*min.*) | Hydrolysis of ε-aminocaproic acid methyl ester ($10^{-3}$ *M*) | |
|---|---|---|
| | Non-treated | Acid-treated |
| 30 | 2.10 | 2.34 |
| 60 | 3.87 | 4.23 |
| 90 | 5.13 | 5.13 |
| 120 | 6.39 | 6.57 |
| 150 | 6.57 | 7.83 |
| 180 | 9.68 | 8.78 |
| 210 | 10.26 | 9.18 |

Non-treated: The trypsin preparation (Trypsin II) was dissolved in 0.01 *N* HCl (contained 0.005 *M* $CaCl_2$) and neutralized at once with addition of equal volume of 0.01 *N* NaOH. This mixture was used as the enzyme solution.

Acid-treated: The enzyme was dissolved in 0.01 *N* HCl contained 0.005 *M* $CaCl_2$, and kept at 25° for 24 hours. This was used as the enzyme solution after neutralization with addition of equal volume of 0.01 *N* NaOH.

Each reaction mixture contained 0.05 *M* of the substrate, 9.7 μg./ml. (protein N) of the Trypsin II and 0.067 *M* phosphate buffer (pH 7.8). Incubation temperature, 25°.

It seems of considerable interest to investigate if trypsin can hydrolyse derivatives of δ-amino-*n*-valeric acid ($C_5$), and γ-amino-*n*-butyric acid (C ), since ε-aminocaproic acid is the ω-amino-$C_6$-*n*-amino acid. Several ω-amino-*n*-fatty acid ($C_5$–$C_3$) derivatives and other compounds related to ε-aminocaproic acid were thus tested for their susceptibility trypsin and the results obtained are recorded in Table IV. As noted in the table these compounds were completely resistant to the hydrolytic action of the enzyme. Since the hydrolytic rate of δ-aminovaleryl and γ-aminobutyryl compounds could not be determined by the hydroxamic acid method, the electric alcohol-alkali titration method was employed. It was also found that ornithine ethyl ester and δ-aminovaleric acid methyl ester did not inhibit competitively the tryptic hydrolysis of ε-aminocaproic acid methyl ester, suggesting that these two compounds have little affinity to the enzyme. At any rate, it may be concluded that for a simple synthetic substrate to be that trypsin requires at hydrolysed by trypsin the molecule should bear a free amino group at one end of a five-membered straight chain such as ε-aminocaproyl-, lysyl and arginyl compounds.

TABLE IV

*Action of Trypsin on ε-Aminocaproic Acid Analogous Coumpounds*

| Substrate | pH | Analytical method* | Extent of reaction (%) | | | |
|---|---|---|---|---|---|---|
| | | | 30 | 60 | 120 | 180 (*min.*) |
| ε-Caprolactam | 7.8 | N | 0.01 | 0.02 | 0.03 | 0.03 |
| Adipamide | 7.8 | N | 0.00 | | 0.00 | |
| δ-Aminovaleramide | 7.8 | N | 0.00 | 0.0 | 0.0 | 0.0 |
| δ-Aminovaleric acid methyl ester | 9.2 | E | | <2 | ± | <1 |
| DL-Ornithine ethyl ester | 5.8 | E | ± | ± | ± | ± |
| γ-Aminobutyric acid methyl ester | 9.2 | E | 0 | 0 | 0 | 0 |
| β-Alaninamide | 7.8 | N | 0 | 0 | 0 | 0 |
| β-Alanine methyl ester | 6.4 | H | 0 | 0 | | |

* Analytical method: E, electric alcohol-alkali titration method; the others were described in Table I.

All reaction mixture were contained 0.05 *M* substrate and 9.7 μg./ml (protein N) of Trypsin II.

## DISCUSSION

The study presented in this communication together with the previous work (*12*) indicates that trypsin is able to hydrolyse the ester, amide and peptide bonds of ε-aminocaproyl compounds. It is known that optimum pH of tryptic hydrolysis of α-benzoyl-L-argininamide and α-benzoyl-L-lysinamide, usual synthetic substrates of the enzyme is pH 7.8 (*9*). The optimum pH of the hydrolysis of ε-aminocaproyl compounds was found to be at pH 6–9 (Fig 1). This minor deviation in optimum pH might be due to differences among the ε-aminocaproyl and arginyl or lysyl compounds.

Michaelis constant ($K_m$) for the hydrolysis of the ε-aminocaproyl compound was found to be $7-17\times10^{-3}$ *M*, a value considerably higher than the $K_m$ for the arginyl or lysyl compounds. This indicates that affinity of trypsin to ε-aminocaproyl compounds is smaller than to the arginyl or lysyl compound. It is interesting, in this connection, to note that ε-aminocaproic acid possesses a structure corresponding to α-deaminolysine.

The proteolytic coefficient $k_3$ for tryptic hydrolysis of ε-aminocaproic acid methyl ester is $145\times10^{-3}$ and this value is quite similar to that for L-arginine methyl ester ($150\times10^{-3}$) (*9*). It was shown in the previous work (*6*) that poly-ε-aminocaproyl-α-alanine can be reversibly coagulated by heat and therefore shows two temperature optima in the tryptic hydrolysis. Such phenomena were, however, not observed in the hydrolysis of ε-aminocaproyl-L-alanine, constitutional unit of the polymer, by trypsin.

Detailed information available up to date shows that one δ-aminovaleric acid ester can be slowly hydrolysed by the action of trypsin (*5*) and that ornithine derivatives are completely resistant to the enzyme action (*11*). In the present communication no hydrolysis of the both compounds was observed. It is necessary, therefore, to perform more extensive studies to be sure of this conclusion, although it seems probable that this discrepancy in the tryptic hydrolysis of δ-aminovaleric acid ester might be due to the different analytical methods employed.

## SUMMARY

In studies on the action of trypsin to ε-aminocaproyl compounds it has been shown that the peptides, amide or esters of ε-aminocaproic acid can be partly hydrolysed by the enzyme action. It seems that an enzyme which catalyzes the hydrolysis of these compounds is trypsin itself from the examination of the stability, inhibitions of the enzymes responsible. Optimum pH, $K_m$ and proteolytic coefficient $k_3$ were also determined for the hydrolysis. Several ω-amino-*n*-fatty acid ($C_5$–$C_3$) derivatives, ornithine ethyl ester, ε-caprolactam and adipamide, structurally related to ε-aminocaproic acid derivatives, made completely resistance to the hydrolytic action of the enzyme.

The authors wish to express their hearty thanks to Prof. F. Egami of Nagoya University, Prof. J. Noguchi of this University, and Dr. R. Sato, Osaka University for their kind encouragements throughout the course of this work. The authors are also grateful to Mr. T. Nojiri for his technical assistances.

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# STUDIES ON EGG YOLK PROTEINS

## V. ELECTROPHORETIC AND ULTRACENTRIFUGAL INVESTIGATIONS ON THE HOMOGENEITIES AND SOME PROPERTIES OF α- AND β-LIPOVITELLIN

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(Received for publication, June 10, 1958)

In previous studies (*1–3*), it was found that two kinds of lipoproteins, termed α- and β-lipovitellin, exist in egg yolk, and the isolations of these lipoproteins and their contents in yolk proteins were reported. Both of them represented a single peak in buffer of pH 9.8, but further informations on their homogeneities were reserved for the following study.

This paper deals with more detailed studies upon the homogeneities and some properties of α- and β-lipovitellin. In the pH range from 4.9 to 10.5, β-lipovitellin has shown a single peak on electrophoresis and an isoelectric point at pH 5.9. Ultracentrifugation at pH 9.8 has represented the heterogeneity of both lipoproteins. The variation of the mobility of β-lipovitellin produced by a change in the buffer composition at constant ionic strength has reflected differences in the binding of the various buffer ions to the protein. The influences of pH and ionic strength on the solubility of β-lipovitellin have been studied.

### EXPERIMENTALS

*Materials*—The α- and β-lipovitellin were prepared by the method reported in an earlier paper (*2*).

*Electrophoresis*—Electrophoresis was carried out in a Tiselius apparatus with a cylindrical lens-diagonal slit optical system. Macro cell (10 ml.) was used through all experiments. Measurements of pH were made with a Beckman Model G instrument, at temperature near 10°. The protein concentration was determined refractometrically. The specific conductivity of the dialyzed buffer solution was measured at 0°. The mobility was calculated from the boundary velocity in descending limb of the cell.

The composition of the buffers used to determine the mobility was as follows:

| pH | $\mu$ | Buffer salts | pH | $\mu$ | Buffer salts |
|---|---|---|---|---|---|
| 4.2 | 0.15 | $CH_3CO_2Na$-$CH_3CO_2H$ | 8.6 | 0.20 | $Na_2B_4O_7$-$KH_2PO_4$ |
| 4.9 | 0.15 | „ | 9.2 | 0.15 | $Na_2B_4O_7$-$NaOH$ |
| 5.3 | 0.15 | „ | 9.8 | 0.15 | „ |
| 5.8 | 0.30 | $Na_2HPO_4$-$KH_2PO_4$ | 10.5 | 0.15 | „ |
| 6.2 | 0.30 | „ | 9.1 | 0.15 | $NaHCO_3$-$Na_2CO_3$ |
| 6.8 | 0.30 | „ | 9.5 | 0.15 | „ |

| | | | | | |
|---|---|---|---|---|---|
| 7.8 | 0.25 | $Na_2HPO_4$-$KH_2PO_4$ | 9.9 | 0.15 | $NaHCO_3$-$Na_2CO_3$ |
| 8.5 | 0.20 | $NH_4Cl$-$NH_4OH$ | 10.5 | 0.15 | „ |
| 8.8 | 0.20 | „ | 9.6 | 0.15 | glycine-NaOH |
| 9.2 | 0.20 | „ | | | |
| 9.8 | 0.15 | „ | | | |

Of the ionic strength indicated, 0.1 depended on the buffer ions and the residue on NaCl.

*Ultracentrifuge*—Ultracentrifugation was performed at room temperature in a Spinco Model E analytical ultracentrifuge at a speed of 59,760 r.p.m.

*Turbidity*—To 9.5 ml. of the various buffers was added 0.5 ml. of the stock solution of β-lipovitellin having a protein concentration of 2 per cent. The mixed solution had an ionic strength of 0.05 due to the buffer ions and an ionic strength of 0 to 0.45 due to NaCl. After an hour the optical transmission of the solution was determined at 650 m$\mu$ with a Beckman DU spectrophotometer.

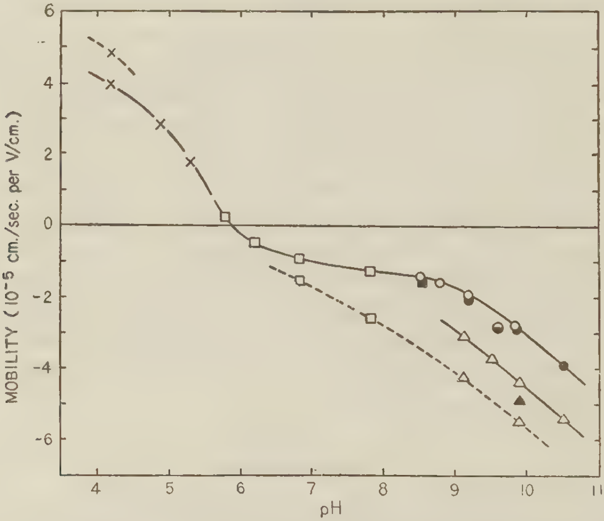

FIG. 1. Mobility curves of α- and β-lipovitellin. The figure also represents the variation of mobility of β-lipovitellin with buffer composition in the range of pH 9.0 to 10.5.

—— β-lipovitellin, ---- α-lipovitellin. × acetate, □ phosphate, ■ phosphate-borate, ○ ammonia, ● borate, △ carbonate, ▲ carbonate+$Na_2SO_4$, ◒ glycine.

## RESULTS

*Mobility Curves and Isoelectric Point*—The pH-mobility curves of α- and β-lipovitellin, obtained in the pH range 4.2 to 10.5, are shown in Fig. 1. The mobilities were measured ordinarily in buffers of 0.15 ionic strength and with a protein concentration of about 0.8 per cent. However, with β-lipovitellin, it was necessary to raise the ionic strength of buffers up to 0.2 to 0.3 between pH 5.8 and 9.0, since the solubility of β-lipovitellin was lowered in this pH range. Such lowering of solubility was observed more excessively with α-lipovitellin. Thus, between pH 6.8 and 9.0, it was compelled to measure the mobility of α-lipovitellin by using the supernatant solution, and, moreover, between pH 4.5 and 6.8 it was unable to obtained the protein concentration which made electrophoresis possible, even at the ionic strength above 0.3. Accordingly, the isoelectric point of α-lipovitellin could not be obtained. On the other hand β-lipovitellin represented the isoelectric point at pH 5.9 (Fig. 1).

β-Lipovitellin showed a single peak in the pH range 4.6 to 10.5, but at pH 4.2 two more minor peaks were observed (Fig. 2-a and b). α-Lipovitellin also showed a single peak in the pH range 6.8 to 9.9, but it constituted two peaks at pH 4.2 (Fig. 2-c and d).

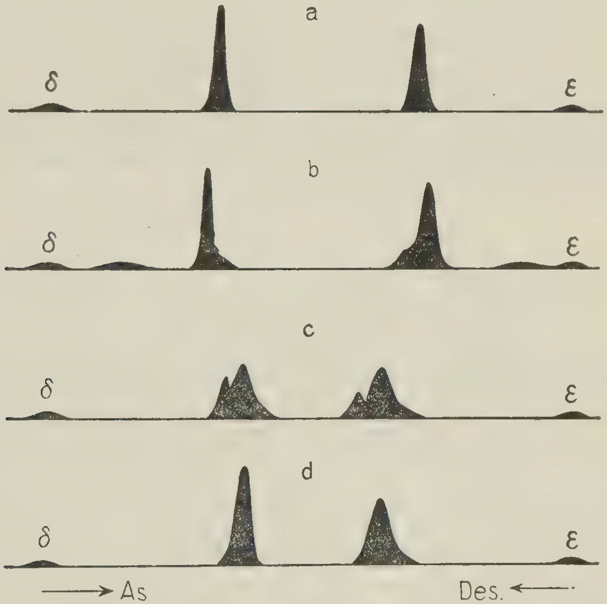

FIG. 2. Electrophoretic patterns of α- and β-lipovitellin: a, β-lipovitellin at pH 9.8 and μ 0.15 (carbonate buffer); b, β-lipovitellin at pH 4.2 and μ 0.15 (acetate buffer); c, α-lipovitellin at pH 4.2 and μ 0.15 (acetate buffer); d, α-lipovitellin at pH 9.8 and μ 0.15 (carbonate buffer).

*Variation of Mobility with Buffer Composition*—It was already reported in several cases that the mobility causes a considerable change with buffer composition and this was attributed to the binding of buffer ions to the protein molecules (*4–6*). β-Lipovitellin also represented such variation of mobility in the pH range from 9.0 to 10.5. The results are shown in Fig. 1. The mobility of β-lipovitellin obtained in carbonate buffer was considerably larger than in both ammonium and borate buffers. In glycine-NaOH buffer, β-lipovitellin gave nearly a similar mobility with that found in ammonium or borate buffer. An effect of sulfate ions was studied by substituting $Na_2SO_4$ for NaCl in the carbonate buffer. The mobility obtained was somewhat larger than the case without substituting.

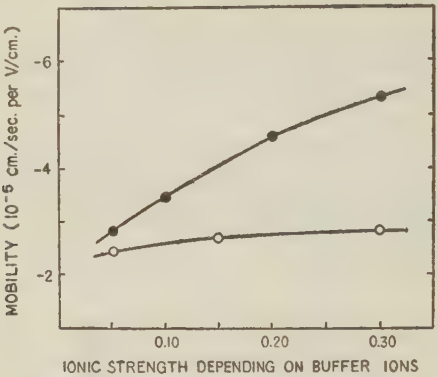

**FIG. 3.** Variation of mobility of β-lipovitellin with buffer composition. The figure also indicates the binding of carbonate ion to β-lipovitellin: ●, carbonate buffer; ○, ammonia buffer; pH 9.9 and total ionic strength 0.30.

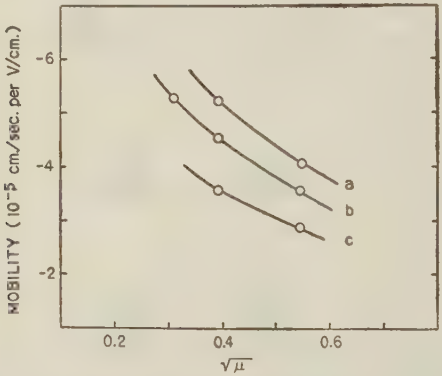

**FIG. 4.** Variation of mobility of β-lipovitellin with ionic strength (in carbonate buffer at pH 9.9). The ionic strength depending on the buffer ions is kept to 0.15 (a), 0.10 (b), and 0.05 (c). (See text).

Another evidence for the binding of carbonate ions is shown in Fig 3. Here, the variation of mobility of β-lipovitellin was studied by changing the ionic strength due to buffer ions from 0.05 to 0.25 while fixing the total ionic strength to 0.3. The obtained mobility gradually increased in carbonate buffer with increase in ionic strength due to buffer ions, whereas not in ammonium buffer. This result also shows that the binding of ammonium ions to β-lipovitellin does not practically occur. Thus, the binding ability to β-lipovitellin could be recognized in the divalent ions such as carbonate and sulfate, but not in the monovalent ions such as ammonium and chloride.

*Dependence of Mobility on Ionic Strength of Buffer*—It is well known that the mobility depends on the ionic strength of buffer (for example, (*5*)). Such dependence was studied with β-lipovitellin by using carbonate buffer. In this case, since the binding of carbonate ions must be considered, measurements of mobility were performed in the buffers whose ionic strength were changed by NaCl. Fig. 4 represents the result obtained in three cases in which the ionic strength due to buffer ions were fixed to 0.05, 0.10 and 0.15, respectively. There was a marked decrease in the mobility of β-lipovitellin with increase in ionic strength. Such a result is expected from the equation of migration velocity (for example, (*7*)), but, in this case, it should also be considered that the binding of carbonate ions to the protein is inhibited more or less by the increase of ionic strength due to NaCl.

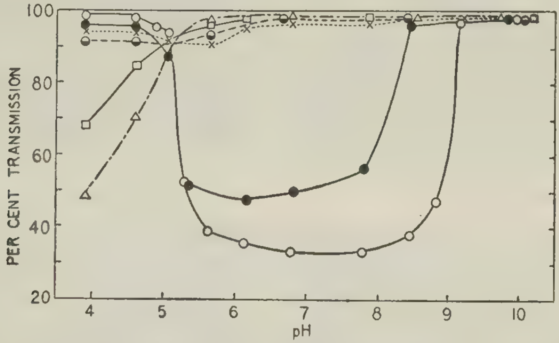

FIG. 5. Solubility property of β-lipovitellin; protein concentration, 0.1 per cent: —○—, 0.05 μ; —●—, 0.10 μ; ···×···, 0.15 μ; - -◒- -, 0.20 μ; —□—, 0.40 μ; —·—△—·—, 0.50 μ (See text).

*Solubility Property of β-Lipovitellin*—In an earlier study (*2*), it was reported that β-lipovitellin is water-soluble at the pH of both acid and basic sides, but not at neutral pH. Here, more detailed study on the solubility of β-lipovitellin was carried out in the pH range from 4.0 to 10.0. The obtained result is shown in Fig. 5. The transparent solution was obtained independently of ionic strength at the pH above 9.0. In the pH range 5.0 to 9.0, the solubility was raised with increase in ionic strength, whereas on the contrary lowered at the pH below 5.0. The pH region showing the minimum solubility shifted progressively to the acid side with increase in ionic strength. At an ionic strength of 0.15, the minimum solubility was found at pH 5.0 to 5.5. This is appreciably lower than the isoelectric point (pH 5.9 at 0.3 ionic strength) obtained by electrophoresis*. A similar disagreement between the isoelectric point and the pH showing the minimum solubility was recently reported in the case of $\alpha$-lactalbumin by Klostergaard and Pasternak (*8*).

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* This difference will become somewhat larger, since it is well known that the isoelectric point more or less shifts to the alkaline side with decrease in ionic strength (*5, 9*).

*Ultracentrifuge Results*—The patterns obtained in carbonate buffer of pH 9.8 and ionic strength 0.15 are shown in Fig. 6. The pattern of $\alpha$-lipovitellin represented a major broad peak ($S\eta_{20,\,w}=17.0$) accompanied by few minor peaks. On the other hand $\beta$-lipovitellin showed two major peaks ($S\eta_{20,\,w}=3.9$ and 12.0) and few minor peaks between them. Evidently, these results indicate that both preparations are heterogeneous ultracentrifugally*.

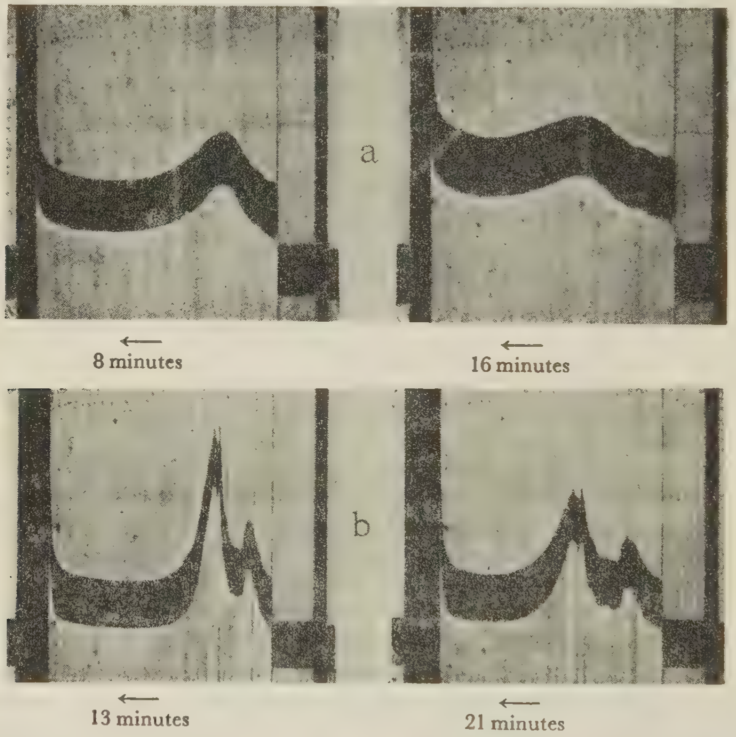

FIG. 6. Sedimentation patterns of $\alpha$- and $\beta$- lipovitellin at pH 9.9 and $\mu$ 0.15 (carbonate buffer). a, $\alpha$-lipovitellin (c = 1.0 per cent) at 59,760 r.p.m.; b, $\beta$-lipovitellin (c = 1.7 per cent) at 59,760 r.p.m.

## DISCUSSION

*Isoelectric Point of $\beta$-Lipovitellin*—Recently, the isoelectric point of so-called lipovitellin has been reported by Young and Phinney (*10*) and Lea and Hawke (*11*). Young *et al.*, using the lipovitellin prepared by the method of Chargaff (*12*), determined the isoelectric point from the point of maximum turbidity and obtained the value of 3.4 to 3.5. Lea *et al.*, using the lipovitellin prepared by their own method (*13*), obtained a value of 5.57 by measuring electrophoresis (microscopic method) in very diluted citrate-phosphate buffer. The latter is fairly close to the value obtained in this work, but, considering the influence of ionic strength on the isoelectric point (*5*, *9*), the difference between both values will become somewhat larger.

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* The pattern of $\beta$-lipovitellin showed an appreciable variation with the condition of medium.

Lipovitellin prepared by the method of Chargaff contained 23 per cent lipid (*12*) and the preparation used by Lea *et al.* contained 28.4 per cent lipid (*11*). As reported by the author (*1*), these materials are considered to be a mixture of $\alpha$- and $\beta$-lipovitellin*. In addition, as their methods used to determine the isoelectric point are different from that used in this work, it is natural that the isoelectric point obtained here do not coincide with the values of the above authors.

*Heterogeneities of $\alpha$- and $\beta$-Lipovitellin*—It has been verified that $\alpha$- and $\beta$-lipovitellin are homogeneous electrophoretically in the pH range 6.8 to 9.9 and 4.9 to 10.5, respectively. However, electrophoresis at pH 4.2 indicated the heterogeneities of both lipoproteins. Several following explanations may be available for the heterogeneities at pH 4.2: (a) several components have a similar mobility with one another at all pH except 4.2; (b) the lipoproteins are unstable in the acid medium; (c) the heterogeneity in particles appears in electrophoresis at pH 4.2. No direct evidence for these explanations was obtained in this experiment.

Further details on the ultracentrifugal studies of $\alpha$- and $\beta$-lipovitellin will be reported in due course.

## SUMMARY

1. Both $\alpha$- and $\beta$-lipovitellin were homogeneous electrophoretically in the pH range 6.8 to 9.9 and 4.9 to 10.5, respectively, whereas at pH 4.2 both were heterogeneous. Ultracentrifugal analysis at pH 9.8 and $\mu$ 0.15 indicated the heterogeneities of both preparations.

2. From the mobility curve, the isoelectric point of $\beta$-lipovitellin was pH 5.9 at an ionic strength of 0.30.

3. From the variation of mobility of $\beta$-lipovitellin with buffer composition, it was pointed out that the divalent anions such as carbonate and sulfate had the binding ability to $\beta$-lipovitellin, but not monovalent ions such as ammonium and chloride.

4. The mobility of $\beta$-lipovitellin depended on the inoic strength of buffer. There was a marked decrease in the mobility of $\beta$-lipovitellin with increase in ionic strength.

5. Some informations on the solubility property of $\beta$-lipovitellin in the pH range from 4.0 to 10.0 were obtained.

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* The lipid contents of $\alpha$- and $\beta$-lipovitellin are 6.5 and 38 per cent, respectively (*2*).

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# STUDIES ON TAKA-AMYLASE A

## VI. ON THE $\alpha$-MALTOSIDASE ACTIVITY OF TAKA-AMYLASE A

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In 1943 Akabori, Shimazu and Nakanishi (*1*) reported that purified Taka-amylase was able to decompose $\alpha$-phenylmaltoside into phenol and maltose. When the enzyme was first crystallized in pure state (*2*), it was reported that Taka-amylase A had no $\alpha$-phenylmaltosidase activity. However, the amount of crystalline enzyme material was limited at that time and exact measurement could not be performed. Recently $\alpha$-phenylmaltoside as obtained in pure crystalline state, it has been made clear that the purified preparation of Taka-amylase A was able to decompose the maltoside to phenol and maltose when considerably high concentrations of the enzyme were used. Present paper deals with the experimental evidence in which Taka-amylase A showed both amylase and $\alpha$-phenylmaltosidase activities, and it was further demonstrated that the determination of Taka-amylase activity with $\alpha$-phenylmaltoside as substrate is a rapid and reliable method.

### METHODS AND MATERIALS

*Taka-amylase A*—Crystalline Taka-amylase A prepared from "Takadiastase, Sankyo" by the method of Akabori *et al* (*2*), was recrystallized three times from aqueous acetone.

*Amylase Activity*—Amylase activity was measured by determining its saccharogenic activity by the method of Fuwa (*3*) with amylose as the substrate at pH 5.3 (acetate buffer).

*Maltosidase Activity*—Maltosidase activity was measured by determining the quantity of phenol produced from $\alpha$-phenylmaltoside by the action of enzyme. 1 ml. of the solution of Taka-amylase and 1 ml. of 0.2 $M$ acetate buffer (pH 5.3) were pipetted into the test tube. After incubation at 37°, 1 ml. of 0.5 per cent $\alpha$-phenylmaltoside solution was added and incubated for 30 minutes. Then 3 ml. of 5 per cent $Na_2CO_3$ were added in the reaction solution to stop the reaction, and 1 ml. of phenol reagent (*4*) was added to the solution and the mixture was kept at 37°. After 20 minutes, the colour density was measured at 660 m$\mu$.

*p*-Nitrophenyl-$\alpha$-maltoside could be used instead of $\alpha$-phenylmaltoside. In this case, *p*-nitrophenol formed was determined colorimetrically at 400 m$\mu$ in alkaline solution without addition of phenol reagent.

*$\alpha$-Phenylmaltoside*—Crystalline $\alpha$-phenylmaltoside was synthesized for the first time by the method of Helferich and Peterson (*5*) who had obtained this compound only

in syrupy state.

Ten grams of heptaacetyl-$\alpha$-phenylmaltoside were dissolved in 100 ml. of a mixture of anhydrous methanol and chloroform (1 : 1, by volume), and saturated with ammonia gas under cooling. After the reaction mixture was kept more than two hours at room temperature, the methanol and ammonia were removed under reduced pressure and the syrupy residue was dissolved in a small quantity of anhydrous ethanol and then anhydrous ether was added to it. The precipitate was washed with ether. This crude crystalline $\alpha$-phenylmaltoside was crystallized from ethanol. The yield is 4.75 g.; m. p. 202-204°; $[\alpha]_D^{14.5}$ 211° (C 1.3, in $H_2O$). Found: C, 51.20; H, 6.19. Calcd. for $C_{18}H_{26}O_{11}$: C, 51.67, H, 6.22 per cent.

*p-Nitrophenyl-α-maltoside*—*p*-Nitrophenyl-$\alpha$-maltoside was synthesized and crystallized by the following procedure: In a 100 ml. Erlenmeyer flask 5 g. of heptaacetyl-$\alpha$-phenylmaltoside were dissolved in the mixture of 20 ml. of glacial acetic acid and 10 ml. of acetic anhydride. Under cooling a mixture of 25 g. of concentrated sulfuric acid (95 per cent) and 25 g. of glacial acetic acid was added. Then a mixture of 5 g. of nitric acid (sp. gr. 1.38) and 10 ml. of glacial acetic acid was added to this solution with stirring. The mixture was kept for two hours at room temperature, and poured into a beaker or flask containing a large amount of ice. Within a few minutes a powdery white precipitate of the heptaacetyl-(*p*-nitrophenyl)-$\alpha$-maltoside separated, which was collected, washed perfectly with water and dried. The yield of the crude substance was 4.83 g. This was crystallized from absolute ethanol containing 10 per cent of dioxane. The yield of pure, crystalline product was 4.75 g. (92 per cent of theoretical amount); m.p. 147-150°. Found: C, 50.61; H, 5.17; N, 1.84. Calcd. for $C_{30}H_{39}O_{10}N$: C, 50.70; H, 5.19; N, 1.85 per cent.

Heptaacetyl-(*p*-nitrophenyl)-$\alpha$-maltoside was deacetylated by the same method as with $\alpha$-phenylmaltoside acetate. The obtained crude product was recrystallized from ethanol. The yield of pure, crystalline *p*-nitrophenyl-$\alpha$-maltoside was 4.25 g.; m.p. 135-8°; $[\alpha]_D^{15}$ 281° (C 6, in water). Found: C, 46.82; H 5.38; N, 3.04: Calcd. for $C_{18}H_{25}O_{13}N$: C, 46.65; H, 5.40; N, 3.02 per cent.

## RESULTS

A comparison of amylase with maltosidase activities of Taka-amylase A in each recrystallization step, and of the crystals obtained from different batches of "Takadiastase, Sankyo", are shwon in Table I. It was found that the ratio amylase activity/$\alpha$-phenylmaltosidase activity of Taka-amylase A remained unchanged even after recrystallization and was also the same

TABLE I

*The Ratio of Amylase- to Maltosidase- Activity of Taka-Amylase A (TAA)*

| Sample | Ratio of amylase to maltosidase activity |
|---|---|
| Once crystallized TAA [VIII] | 1.11 |
| Thrice „ „ „ | 1.03 |
| Twice „ „ [XI] | 1.03 |
| Dialized TAA incubated with EDTA (none $Ca^{++}$) | 0.97 |
| 13 days, 37°, incubate TAA | 1.16 |

with different enzyme batches of "Taka-diastase". Tables II and III show the ratio of amylase activity to $\alpha$-phenylmaltosidase activity of partially denatured TAA either by the heat treatment or by various denaturing

TABLE II

*The Ratio of Amylase- to Maltosidase- Activity of Partially Denatured TAA after Heat Treatment*

| Temp. (°C) | Time (*min.*) | pH | Added substances | Remained enzyme activity | | (A)/(M) |
|---|---|---|---|---|---|---|
| | | | | Amylase (A) | Phenyl-maltosidase (*M*) | |
| 60 | 20 | 7.0 | 0.05 *M* Ca | 85 (%) | 94 (%) | 0.91 |
| ” | ” | ” | — | 77 | 92 | 0.81 |
| ” | ” | 5.0 | 0.05 *M* Ca | 85 | 94 | 0.91 |
| ” | ” | ” | — | 11 | 16 | 0.69 |
| ” | 10 | ” | Amylose | 85 | 88 | 0.97 |
| ” | ” | ” | $H_2O$ | 72 | 71 | 1.01 |

TABLE III

*The Ratio of Amylase to Maltosidase of Partially Denatured TAA with Various Denaturing Agents*

| Reagent | Time | Remained enzyme activity | | (A)/(M) |
|---|---|---|---|---|
| | | Amylase (A) | Phenyl-maltosidase (M) | |
| $H_3PO_4$1) | 30 (*min.*) | 82 (%) | 88 (%) | 0.93 |
| | 60 | 66 | 73 | 0.92 |
| | 120 | 43 | 47 | 0.92 |
| | 210 | 25 | 30 | 0.83 |
| E.D.T.A.2) | 5 (*hrs.*) | 42 | 54 | 0.78 |
| | 20 | 13 | 20 | 0.65 |
| D.N.B.S.3) | 20 (*hrs.*) | 81 | 89 | 0.91 |
| | 69 | 67 | 72 | 0.93 |
| Urea4) | 1 (*hrs.*) | 84 | 75 | 1.12 |
| | 2 | 68 | 50 | 1·36 |
| | 4 | 48 | 39 | 1.23 |

Conditions of denaturation are as follows:

1) 0.1 *M* phosphoric acid, at 16°, pH 2.4. After two hours, added $NaHCO_3$, and then amylase and phenylmaltosidase activities were recovered till 80 per cent and 100 per cent. But after three hours they were not recovered.

2) 200 *M* EDTA per one mole TAA were added in the enzyme solution of 0.05 *M* veronal buffer at 37°, pH 7.0.

3) 100 *M* DNBS per 1 *M* TAA were added in the enzyme solution at 15°, pH 10.4.

4) In 8 *M* urea solution, at 37°, pH 6.0.

agents—acid, ethylenediamine tetraacetate (EDTA), dinitrobenzene sulfonate (DNBS), and urea. The ratio of remaining activity, amylase/$\alpha$-phenylmaltosidase was about the same in both cases, but when the inactivation of Taka-amylase A occurred very strongly, the decrease in amylase activity was more intense than the decrease in maltosidase.

Therefore, it is supposed that the whole steric configuration of the enzyme protein molecule as a whole may be responsible for acting upon high molecular substrates such as starch or amylose, whereas that of one particular part of the enzyme protein is very important to decompose lower molecular substrates as $\alpha$-phenylmaltoside, the other part of the molecule being less important than in the case of high molecular substrates. Fig. 1 shows the pH-dependence of amylase and $\alpha$-phenylmaltosidase activities of crystalline Taka-amylase A. The optimum pH of the both activities was between pH 5 and 5.5, and the shapes of the pH-activity curves were similar.

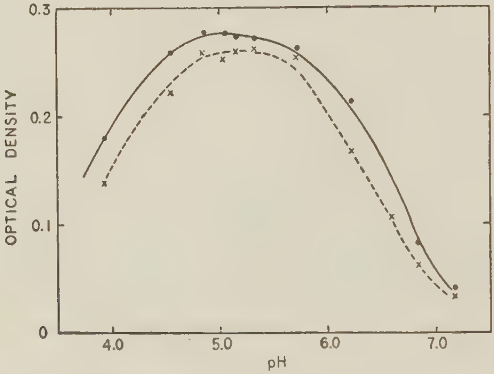

**FIG. 1.** pH-activity curve of Taka-amylase A.
—●— amylase activity, --×-- maltosidase activity.

For the purpose to confirm whether the $\alpha$-phenylmaltosidase activity will be an intrinsic property of Taka-amylase A or will be caused by the contamination of the different enzyme "Taka-maltosidase", the authors tried to separate these two activities by means of vertical starch column electrophoresis as follows: Using a column (1.8×29 cm.) of potato starch, 6.7 mg. of crystalline Taka-amylase A was subjected to electrophoresis; 200 v., 10 mA in 0.1 *M* acetate buffer pH 6.0, ionic strength 0.05, 15 hours at room temperature. After electrophoresis, the column was set on the fraction collector, eluted with 0.1 *M* acetate buffer and fractionated into each 1 ml. fractions.

The protein concentration and amylase, maltosidase, and maltase activities of each fraction were determined. Attempts to separate these two activities failed as shown in Fig. 2.

It can be seen that the peak of protein as spectrophotometrically measured (optical density at 280 m$\mu$) closely coincided with the peaks of both amylase and $\alpha$-phenylmaltosidase activities. But it was found that the small peak of protein eluted later than TAA had no activities of amylase and maltosidase, but maltase activity.

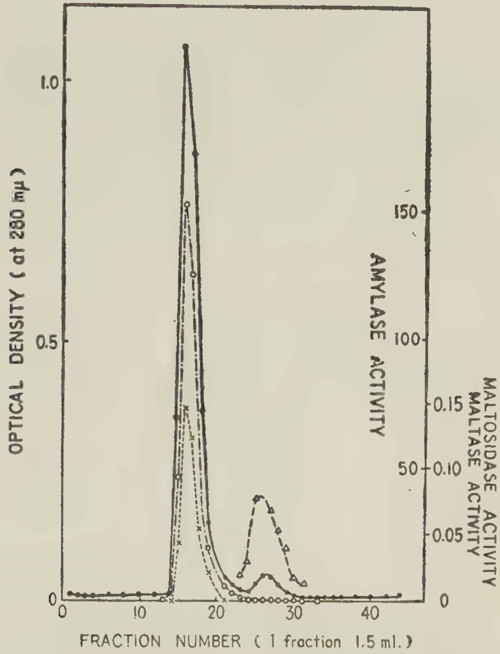

**FIG. 2.** Starch column-zone electrophoresis to Taka-amylase A.

—·○—· amylase activity, – –×– – maltosidase activity, – –△– – maltase activity, —●— absorption at 280 m$\mu$.

## DISCUSSION

The results described above indicate that the $\alpha$-phenylmaltosidase activity is an intrinsic property of the Taka-amylase A protein itself.

In the studies of the mechanism of enzyme action, it is necessary to measure the enzymatic activity by a method of strict reproducibility. Soluble starch or amylose has been usually empolyed as the substrate in the determination of amylase activity. These natural high polymers are, however, not suitable for exact, scientific research works, because these substrates do not always give reproducible results owing to the inhomogeneity in respect of the molecular weight. Therefore, it is desirable to use an appropriate synthetic substrate possessing a definite composition and substrate of low molecular weight is more convenient than macromolecular substrates of undefined compositions such as starch. From this point of view, it seems to be very convenient to measure the amylase activity with $\alpha$-phenylmaltoside.

$\alpha$-Phenylmaltoside was first synthesized by Helferich and Peterson as a syrupy preparation. We have, however, succeeded in crystallizing this compound and also $p$-nitrophenyl-$\alpha$-maltoside which had not been reported yet. Therefore the enzyme activity of Taka-amylase A could easily be determined by the use of $\alpha$-phenylmaltoside or $p$-nitrophenyl-$\alpha$-maltoside as the substrates, moreover the latter is especially suited to rapid determination. However, the action of Taka-amylase A to $\alpha$-phenylmaltoside was considerably weaker than to amylose. Therefore several hundred times higher concentration of the enzyme was required for the activity measurement with $\alpha$-phenylmaltoside than with amylose as substrate.

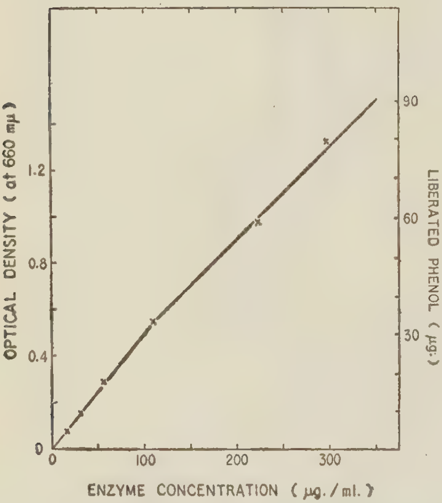

FIG. 3. Measurement of activity of Taka-amylase A using $\alpha$-phenylmaltoside.

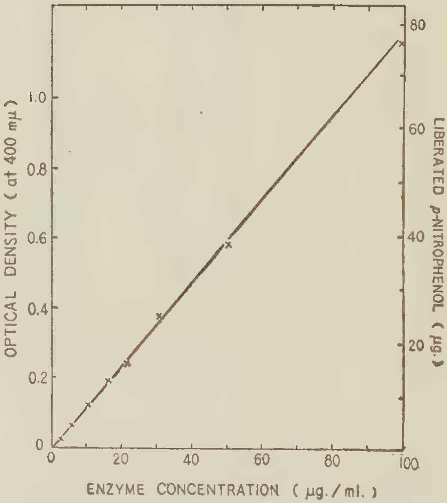

FIG. 4. Measurement of activity of Taka-amylase A using $p$-nitrophenyl-$\alpha$-maltoside.

The use of the extremely dilute solution of enzyme, as in the case of amylase-amylose reaction, may be a cause of error in the determination of enzyme activity, for the denaturation may accompany with the dilution. Therefore, amylase activity could be determined more accurately by the use of $\alpha$-phenylmaltosides.

By measuring the rate of liberation of phenol (or $p$-nitrophenol) from the substrate, it would be possible to measure the activity of Taka-amylase A accurately even if maltase were contaminated. Figs. 3 and 4 show the relation between the quantity of phenol or $p$-nitrophenol produced by the enzyme action of different enzyme concentrations.

A new unit of Taka-amylase A was defined as follows: The amount of $\alpha$-phenylmaltosidase activity is conveniently expressed as the maltosidase unit (M. U.) value. It was defined as the amount of enzyme which hydrolyzes

$\alpha$-phenylmaltoside ($p$-nitrophenyl-$\alpha$-maltoside) in $4\times10^{-3}\,M$ concentration producing 1 $\mu$mole of phenol (or $p$-nitrophenol) in 30 minutes (at 37° and pH 5.3) under the conditions described in Figs. 4 and 5. According to this definition, 1 mg. of crystalline Taka-amylase A corresponds to 2.873 M. U.

## SUMMARY

1. The hydrolysis by crystalline Taka-amylase A of $\alpha$-phenylmaltoside and $p$-nitrophenyl-$\alpha$-maltoside was confirmed and the maltosidase activity of Taka-amylase A was found to be an intrinsic property of this enzyme.

2. The preparation and properties of crystalline $\alpha$-phenylmaltoside and the synthesis of $p$-nitrophenyl-$\alpha$-maltoside were described.

3. The method of determination of Taka-amylase A activity using $\alpha$-phenylmaltoside and $p$-nitrophenyl-$\alpha$-maltoside was described, and the maltosidase unit was proposed as a new measure of Taka-amylase activity.

The authors wish to express their gratitude to Mr. A. Tsugita, T. Matsushima, K. Yamamoto and Miss H. Toda for their helpful advice through this investigation, and also to Sankyo Co., Ltd. for their kind supply of "Takadiastase Sankyo".

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The Journal of Biochemistry, Vol. 46, No. 4, 1959

# INTERRELATION BETWEEN THE FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

## X. ON THE REACTION OF REDUCED HEMOGLOBIN WITH ETHYLISOCYANIDE AND THE EFFECT OF UREA ON THIS REACTION

By TARO OKAZAKI AND KEIZOO TSUSHIMA

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(Received for publication June 25, 1958)

The reaction of reduced hemoglobin with ethylisocyanide (EIC) has been studied by Warburg (*1*), Russel (*2*), St. George and Pauling (*3*). Based on their experimental results obtained in the combining reaction of hemoglobin with various alkylisocyanides, St. George and Pauling have proposed a steric hindrance theory of the mechanism of heme-heme interaction in the reactivity of hemoglobin molecule.

The present paper deals with quantitative studies on the reaction of reduced hemoglobin with EIC and the effect of urea on this reaction. The purpose of the present investigations is to re-examine the mechanism of the heme-heme interaction and the Bohr effect of hemoglobin which have so far been observed in the reaction of hemoglobin with oxygen. As is well known, these two effects are of the paramount importance in the chemistry of respiration.

### EXPERIMENTALS

EIC was synthesized from silver cyanide and ethyliodide by the method of Gautiér (*4*).

Hemoglobin solution was prepared as follows: Crystalline equine oxyhemoglobin prepared by the method of Suzuki *et al.* (*5*) was dissolved in appropriate buffer solutions and reduced with sodium dithionite immediately before use.

Myoglobin solution was prepared by dissolving a crystalline preparation of metmyoglobin followed by addition of a small amount of sodium dithionite. Crystalline metmyoglobin was prepared by the method of Tsushima *et al.* (*6*) from horse heart muscle.

Concentrations of hemoglobin and myoglobin solutions were determined by the alkali-denatured globin hemochrome method and cyan-methemoglobin method. The urea used was a commercial preparation.

All reactions were carried out at room temperature in a cuvette and measured spectrophotometrically by means of a photoelectric spectrophotometer Model EPB-V of Hitachi Co.

pH of the solutions were measured by using a glass electrode pH meter of Tooyoo-rika Co.

## RESULTS

*I. Reaction of Hemoglobin with EIC at Definite pH*—The absorption figure of reduced hemoglobin converts to that of EIC-hemoglobin on addition of EIC in an excessive concentration. Fig. 1 shows the absorption figures of hemoglobin in the presence of EIC in various concentrations. In this figure, clear isosbestic points at 542, 549 and 568 mμ were demonstrated indicating a reaction system of two components. As there occurred no further absorption change above $1.32\times10^{-3}$ *M* of the EIC concentration, the absorption figure of Fig. 1—V may be taken as that of the final saturated state of EIC-hemoglobin. Taking this absorption figure as that of the final state, the percentage of EIC-hemoglobin formation at each EIC concentration can be calculated. Plotting these values against the log concentrations of uncombined EIC gave a sigmoid curve of a high order reaction as shown in Fig. 2.

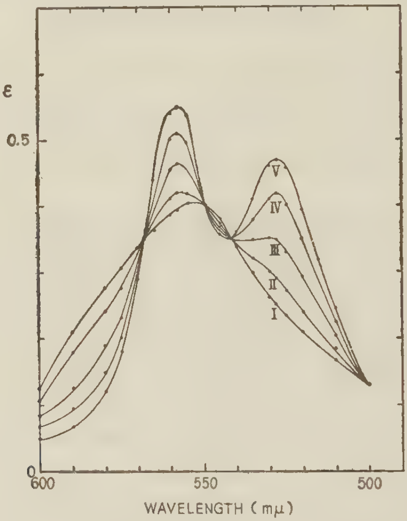

FIG. 1. Absorption changes of reduced hemoglobin caused by the addition of EIC (pH 6.8) at 25°.

Hemoglobin concentration; $3.0\times10^{-5}$ *M*, EIC concentrations; I, 0 *M*; II, $4.85\times10^{-5}$ *M*; III, $7.78\times10^{-4}$ *M*; IV, $1.32\times10^{-3}$ *M*; $2.64\times10^{-3}$ *M*.

By introducing the data obtained in the present experiment to Hill's equation *(7)*

$$Y=K_p{}^n/1+K_p{}^n$$

which has been proposed for the reaction of reduced hemoglobin with oxygen, the sigmoid coefficient of the symmetrical sigmoid curve shown in Fig. 2 was calculated to be 2.4. It is thus proved that there exists a heme-

heme interaction also in the reaction of reduced hemoglobin with EIC, just as in the reaction of hemoglobin with oxygen. The sigmoid coefficient of the reaction of reduced hemoglobin with oxygen has been reported to be 2.8. The difference of the sigmoid coefficient in these two reactions may be attributed to the difference of the ligands.

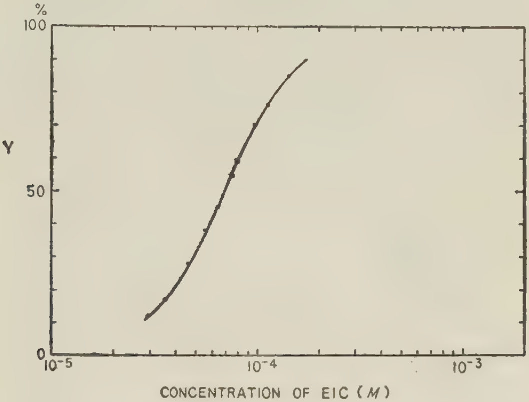

**Fig. 2.** EIC dissociation curve of horse hemoglobin (pH 6.8) at 25°. Hemoglobin concentration; $3.0 \times 10^{-5}$ *M*.

*II. Effect of pH on the Reaction of Hemoglobin with EIC*—The percentages of EIC-hemoglobin formation were plotted against log concentrations of EIC at various pH as shown in Fig. 3. Each of the symmetrical sigmoid curves has the same sigmoid coefficient of 2.4. Fig. 4 shows the relationship between EIC concentrations required for the half formation of EIC-

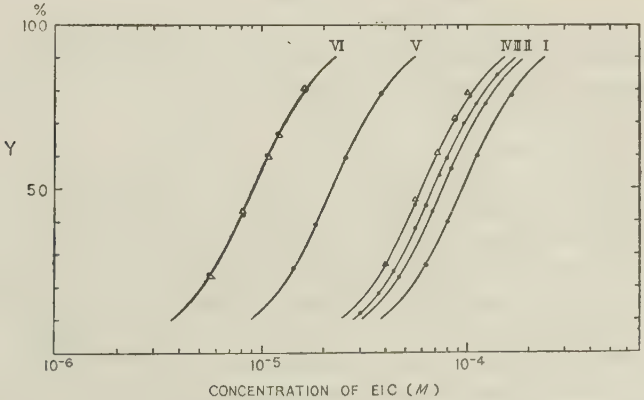

Fig. 3. EIC-dissociation curves of horse hemoglobin in various pH at 25°.

Hemoglobin concentration; $3.0 \times 10^{-5}$ *M*. pH; I, 6.2; II, 5.5; III, 6.8; IV, 5.0, 7.0; V, 8.0; VI, 8.5, 9.0.

hemoglobin (designated as $Y_{50}$) and pH. The curve in Fig. 4 is a theoretical curve calculated by introducing the values of $pK_1$ and $pK_2$ of heme linked acid groups of oxyhemoglobin, which were obtained by Wyman (*8*), to the following equation derived by Wyman (*8*).

$$\log p_{\frac{1}{2}} = \text{constant} + \log\{(H^+ + K_1') (H^+ + K_2')/(H^+ + K_1'') (H^+ + K_2'')\}$$

$$pK_1', 6.68;\ pK_2', 5.75;\ pK_1'', 7.93;\ pK_2'', 5.25.$$

Experimental data coincide closely with the theoretical curve. From this result, the values of $pK_1$ and $pK_2$ of EIC-hemoglobin are assumed to be the same as those of oxyhemoglobin. Fig. 4 represents a graphical expression of the Bohr effect.

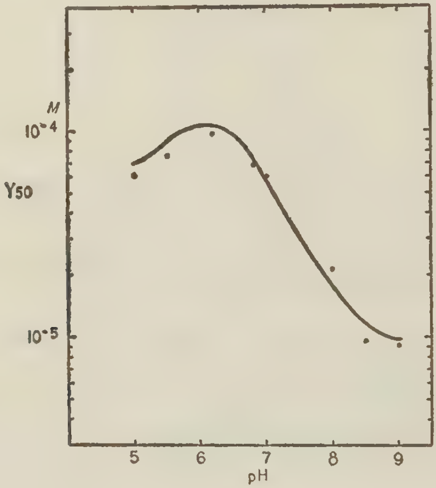

FIG. 4. pH dependence of $Y_{50}$.

*III. Reaction of Reduced Myoglobin with EIC and the Effect of pH*—Myoglobin has been reported to have no Bohr effect (*9*). Therefore, to test the effect of pH on the EIC combining reaction of myoglobin, the following experiments were carried out.

On the addition of EIC in an excessive concentration the absorption figure of reduced myoglobin converts to that of EIC-myoglobin as shown in Fig. 5—V. This absorption figure is slightly different from that of EIC-hemoglobin. Clear isosbestic points were observed at 544, 556 and 575 m$\mu$. In Fig. 6, the percentages of EIC-myoglobin formation were plotted against the log concentrations of EIC. As may be seen, a sigmoid curve was obtained which was symmetrical and of a first order. No change of EIC-binding affinity was observed in the range of pH from 6.2 to 8.0.

These results indicate that neither the heme-heme interaction nor the pH effect exists in the reaction of reduced myoglobin with EIC, just as in the reaction of myoglobin with oxygen.

It was proved from the above experiments that the reaction of reduced hemoglobin with EIC may be used for studies of the function of hemoglobin instead of the reaction of hemoglobin with oxygen.

Thus, for the purpose of studying the relationship between the characteristic functions of hemoglobin, the heme-heme interaction and the Bohr

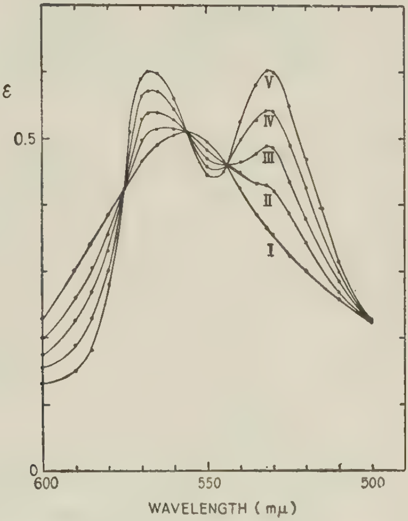

FIG. 5. Absorption changes of reduced myoglobin caused by the addition of EIC (pH 6.8) at 15°.

Myoglobin concentration; $4.25 \times 10^{-5}$ *M*. EIC concentrations; I, 0 *M*; II, $1.95 \times 10^{-5}$ *M*; III, $4.33 \times 10^{-5}$ *M*; IV, $9.15 \times 10^{-5}$ *M*; V, $1.89 \times 10^{-3}$ *M*, $3.78 \times 10^{-3}$ *M*.

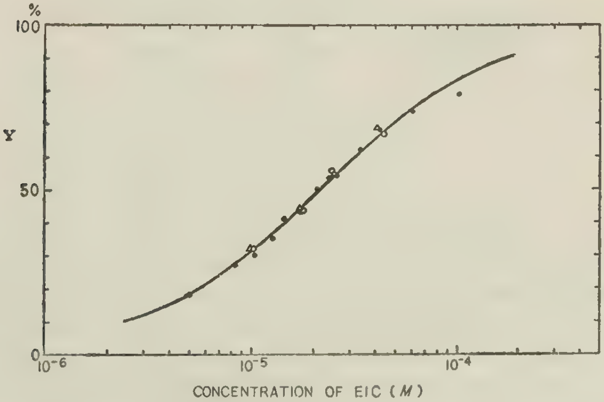

FIG. 6. EIC dissociation curve of myoglobin in various pH at 15°.

Myoglobin concentration; $4.25 \times 10^{-5}$ *M*. pH. —●—, 6.8; —○— 8.0; —△— 6.2.

effect, and the structural modification of its protein part, the following experiments on the reaction of hemoglobin with EIC were carried out.

*IV. Effect of Urea on the Reaction of Reduced Hemoglobin with EIC*—The absorption figure of EIC-hemoglobin in 6.0 *M* urea is the same as that of EIC-hemoglobin in the absence of urea.

The percentages of EIC-hemoglobin formation were plotted against log concentrations of EIC as shown in Fig. 7, giving a symmetrical sigmoid curve having a sigmoid coefficient of 1.4. The data recorded in Table I show that the EIC-combining affinity increases, and the heme-heme interaction decreases, in the presence of 6.0 *M* urea.

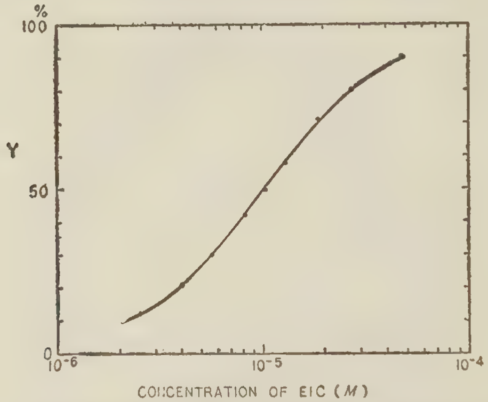

**FIG. 7.** EIC dissociation curve of hemoglobin in the presence of 6.0 *M* urea at 20°.

Hemoglobin concentration; $3.25\times10^{-5}$ *M*. Urea concentration; 6.0 *M*. pH; 6.85.

TABLE I

| Concentrations of urea (*M*) | $Y_{50}$ (*M*) | n |
|---|---|---|
| 0 | $7.0\times10^{-5}$ | 2.4 |
| 6.0 | $1.0\times10^{-5}$ | 1.4 |

pH; 6.85.

The percentages of EIC-hemoglobin formation in various pH were plotted against log concentrations of EIC as shown in Fig. 8.

The curve represented here is a theoretical curve with a sigmoid coefficient of 1.4 and $Y_{50}$ of $1.0\times10^{-5}$ *M*. This value nearly coincides with that obtained above pH 8.5 in the absence of urea (from Fig. 4). Fig. 8 shows that even in the presence of 6.0 *M* urea, there occurs no change of

EIC combining affinity with the pH change in the range from 5.30 to 9.30.

In the presence of 2.0 *M* urea, each of the sigmoid curves obtained in various pH has the same sigmoid coefficient of 2.4, which differs from the values obtained in the presence of 6.0 *M* urea. As shown in Table II, EIC combining affinity of reduced hemoglobin in the presence of 2.0 *M* urea is higher than that in the absence of urea. Fig. 9 shows the relationship between $Y_{50}$ and pH in the presence and absence of urea. It is evident from these results that the heme-heme interaction of hemoglobin is not affected by the addition of 2.0 *M* urea, while the Bohr effect is profoundly affected by the same concentration of urea.

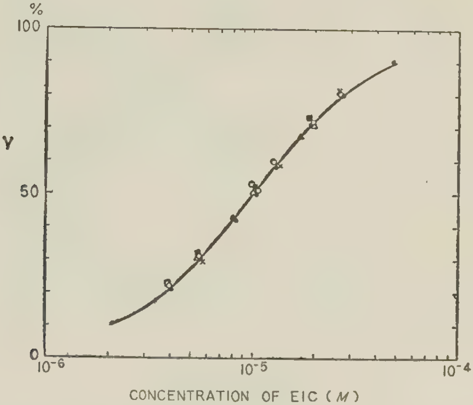

**FIG. 8.** EIC dissociation curve of hemoglobin in 6.0 *M* urea in various pH at 20°.

Hemoglobin concentration; $3.25\times10^{-5}$ *M*. Urea concentration; 6.0 *M*. pH: —■—, 5.30; —×—, 5.80; —▲—, 6.60; —●—, 6.85; —△—, 7.90; —○—, 8.45; —□—, 9.30.

TABLE II

| Concentrations of urea (*M*) | $Y_{50}$ (*M*) | n |
|---|---|---|
| 0 | $1.0\times10^{-4}$ | 2.4 |
| 2.0 | $3.8\times10^{-5}$ | 2.4 |

pH; 6.15.

The data presented in Table III show that $Y_{50}$ decreases continuously with the increase of urea concentration, while the sigmoid coefficient remains constant in the concentrations of urea below 2.0 *M*. It decreases abruptly, however, on the addition of urea in higher concentrations.

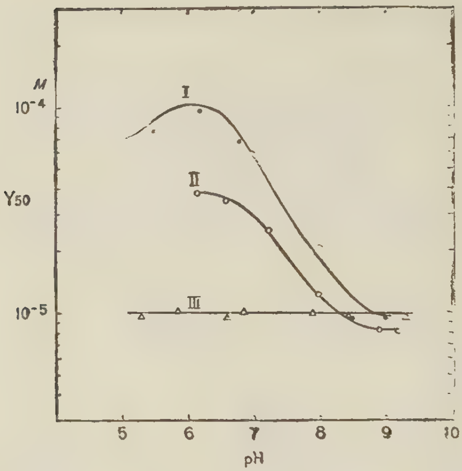

FIG. 9. pH dependence of $Y_{50}$ in various concentrations of urea.

Urea concentrations; I, 0 *M*; II, 2.0 *M*; III, 6.0 *M*.

TABLE III

| Concentrations of urea (*M*) | $Y_{50}$ ($\times 10^{-5}$ *M*) | n |
|---|---|---|
| 0 | 9.00 | 2.4 |
| 0.5 | 7.40 | " |
| 1.0 | 5.90 | " |
| 1.5 | 4.40 | " |
| 2.0 | 3.40 | " |
| 2.5 | 2.80 | 1.7 |
| 3.0 | 2.25 | 1.5 |
| 4.0 | 1.40 | 1.4 |
| 5.0 | 1.00 | 1.4 |

pH; 6.0.

## DISCUSSION

I) As mentioned above, in the presence of 6.0 *M* urea the sigmoid coefficient in the combining reaction of reduced hemoglobin with EIC was calculated to be 1.4. From this value it may be assumed that in the presence of 6.0 *M* urea, the heme-heme interaction persists decisively in the hemoglobin molecule, though only slightly. This result coincides with that obtained by Wyman (*8*) for the reaction of reduced hemoglobin with oxygen.

The decrease of heme-heme interaction of hemoglobin in the concentrated urea solution has been assumed to be caused by the splitting of hemoglobin into half molecules.

The data obtained from our experiments on dog hemoglobin (Table IV and Fig. 10) show that heme-heme interaction, EIC affinity of hemoglobin and the Bohr effect are all affected decisively by the addition of 6.0 $M$ urea, despite of the report that the dog hemoglobin does not split into half molecules (*10*). Furthermore, the increase of EIC-combining affinity of dog hemoglobin is observable in the presence of urea even in its lower concentrations (0.5–2.0 $M$).

TABLE IV

| Concentrations of urea ($M$) | $Y_{50}$ ($M$) | n |
|---|---|---|
| 0 | $2\ 55\times10^{-5}$ | 2.4 |
| 6.0 | $4.20\times10^{-6}$ | 1.4 |

pH; 7.0.

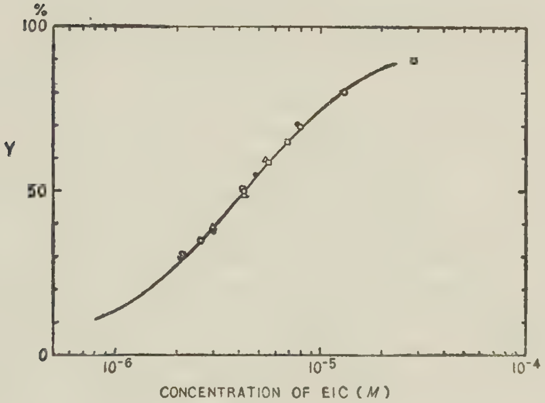

FIG. 10. EIC dissociation curve of dog hemoglobin in 6.0 $M$ urea.

Urea concentrations; 6.0 $M$. pH: —□—, 6.0; —●—, 6.5; —○—, 7.5; —△—, 8.5. Temperature; 14°

Based on these results it will be assumed that the decrease in heme-heme interaction and the increase of EIC combining affinity are not necessarily a result of the splitting of the hemoglobin molecule.

As shown in Table III, $Y_{50}$ in pH 6.0 decreases continuously with the increase of urea concentrations (from 0.5 to 5.0 $M$). However, no change of sigmoid coefficient is observed in the lower concentrations of urea, namely, beneath 2.0 $M$. From these results it will be assumed that there exists no definite relationship between the heme-heme interaction and the EIC-combining affinity.

It is apparent in any case, that even through the structural modification of the hemoglobin molecule in the slightest grade in which the heme-heme interaction is still maintained, the affinity for EIC is decisively altered. This would suggest that the process of structural modification of the hemoglobin molecule is of a successive character.

II) In respect to the mechanism of the Bohr effect, it has been considered that dissociations of heme-linked acid groups are linked directly to the affinity of heme iron of hemoglobin for oxygen.

As proved in the present experiments, there is no Bohr effect in the presence of 6.0 *M* urea in the reaction of reduced hemoglobin with EIC. For this result two possible elucidations may be proposed. (a) The dissociation of heme-linked acid groups will be changed profoundly by the addition of urea. (b) The linkage of acid groups with heme iron will be disrupted through the modification of globin moiety by urea.

The affinity of reduced hemoglobin for EIC in the presence of 6.0 *M* urea is the same in every pH and similar to that above pH 8.5 in the absence of urea. This fact suggests that profound changes of dissociation constants of heme-linked acid groups (these groups have been assumed to be imidazole groups) may occur in the presence of 6.0 *M* urea. However, by considering our other experimental results which show only a slight change of dissociation constant of NH group of free histidine in the presence of 6.0 *M* urea, it is considered improbable that in the presence of urea, dissociation constants of heme-linked acid groups might be strongly altered. Accordingly, the possibility of the first elucidation will be excluded and the second elucidation would remain as a possibility. If it is assumed that the heme-linked acid groups link with heme iron only through the native globin structure, the disappearance of the Bohr effect in the presence of concentrated urea may be explained as a result of structural modifications of globin moiety

## SUMMARY

1. Sigmoid coefficient in the reaction of reduced hemoglobin with EIC was calculated to be 2.4 from Hill's equation. This result indicates the existence of heme-heme interaction in the reaction of reduced hemoglobin with EIC.

2. Dissociation constants of heme-linked acid groups of EIC-hemoglobin were assumed to be similar to those of oxyhemoglobin.

3. In the presence of 6.0 *M* urea, the heme-heme interaction decreased (n decreased from 2.4 to 1.4), EIC-combining affinity increased and the Bohr effect disappeared.

In the presence of urea in lower concentrations, the heme-heme interaction did not decrease and the EIC-combining affiinity increased slightly.

4. The Bohr effect disappeared continuously with the increase of urea concentration.

Some discussions were made on the mechanism of the Bohr effect.

The authors are indebted to Prof. K. Kaziro for his continuous help and stimulating discussions. The authors also express their gratitude to the Ministry of Education for assistance from the Scientific Research Fund.

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# STUDIES ON THE ASYMMETRICAL HYDROLYSIS OF $N^{\alpha}$, $N^{\varepsilon}$-DIACYL-DL-LYSINE*

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(Received for publication, August 20, 1958)

It has been reported by Kameda *et al.* (*1*) that three different kinds of acylases capable of performing the resolution of acyl-DL-amino acids were produced from a *Pseudomonas* species, KT-83, which was previously isolated from soil (*2*), and that two of those acylases hydrolyze asymmetrically the acyl groups at $\alpha$- and $\varepsilon$-position of $N^{\alpha}$, $N^{\varepsilon}$-dibenzoyl-DL-lysine, and the third one hydrolyzes the acyl group of $N^{\alpha}$-benzoyl-D-phenylalanine.

Whereas there have been published many papers dealing with the isolation of the $\alpha$-lysine acylases from *Aspergillus* species (*3*), hog kidney (*4*) and other sources (*5*), only one report has been so far available on the $\varepsilon$-lysine acylase by Paik *et al.* (*6*) who obtained it from rat kidney.

In contrast to the fact that these acylases are able to hydrolyze acetyl-DL-amino acids, and are practically ineffective upon benzoyl-DL-amino acids, the acylases prepared from KT-83 asymmetrically hydrolyze benzoyl-DL-amino acids in preference to acetyl-DL-amino acids.

The author has undertaken the fractionation of the crude $\varepsilon$-lysine acylase of KT-83 (supplied by Kameda) and has obtained the enzyme preparation as four thousand fold active as the original enzyme solution. The highly purified enzyme possesses only $\varepsilon$-lysine acylase activity. A systematic study of the enzymatic activity at various stages of purification has clarified the mechanism of the conversion by KT-83 of $N^{\alpha}$, $N^{\varepsilon}$-diacyl-DL-lysine to L-lysine and unchanged $N^{\alpha}$, $N^{\varepsilon}$-diacyl-D-lysine.

## EXPERIMENTAL

*Enzyme Studies*— The assay of the $\alpha$-lysine acylase was carried out using $N^{\alpha}$, $N^{\varepsilon}$-dibenzoyl-DL-lysine as substrate and by means of the ninhydrin method described by Moore and Stein (*7*).

For the assay of the $\varepsilon$-lysine acylase, a number of experiments were made to determine the most suitable conditions for the differential analysis of $N^{\varepsilon}$-acyl-DL-lysine and L-lysine. This determination was successfully achieved based on the fact that the development of Chinard's colour (*8*) was specific for lysine but negative with $N^{\varepsilon}$-acyl-DL-lysine. A 0.02 $M$

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* This work was presented at the 31st Annual Meeting of the Japanese Biochemical Society held in Sapporo, in July, 1958.

solution of $N^{\varepsilon}$-acyl-DL-lysine was incubated with an equal volume of the enzyme preparation dissolved in a 0.02 *M* phosphate buffer at pH 6.0. After one hour, 0.2–0.4 ml. of the incubated solution was added to 1.0 ml. of glacial acetic acid and 1.0 ml. of a 2 per cent solution of ninhydrin in glacial acetic acid, and the tube was immersed in boiling water for one hour. The solution was diluted with 7.0 ml. of glacial acetic acid, and the absorption intensity was measured at 500 mμ, giving a measure of the free lysine produced by the enzymatic hydrolysis.

*Growth Conditions and Purification of the ε-Lysine Acylase of KT-83*—KT-83 was inoculated into a bouillon culture medium at pH 7.2, and the mixture was maintained for 24 hours at 25° under adequate aeration and agitation. The yield of the cells in a wet state was approximately 150–200 g. per 10 liters of medium. The cells harvested by centrifugation were washed, ground with alumina and then extracted with 600 ml. of a 0.02 *M* phosphate buffer at pH 7.8. To the extract was added 300 ml. of a 2 per cent solution of rivanol (2-ethoxy-6,9-diaminoacridine lactate), whereupon precipitation occurred. After removal of the precipitate and excess rivanol in the solution, the enzyme in the solution was adsorbed on 100 ml. of Amberlite XE-64 at pH 5.0 and was eluted from the resin by adjusting the pH to 7.8 with a 1.0 *M* potassium hydroxide solution. After treatment of the eluate, con-

TABLE I

*Purification of the ε-Lysine Acylase*

| Stage of purification | Total protein | ε-Lysine acylase activity* | Yield | Specific activity | α-Lysine acylase activity* |
|---|---|---|---|---|---|
| | *(mg.)* | *(μM/hr.)* | *(%)* | *(μM/hr./mg. protein)* | *(μM/hr.)* |
| Cells, wet weight. 200 g. | | 157,000 | | | 48,000 |
| Ground with alumina and extracted with buffer at pH 7.8. 400 ml. | 107,200 | 115,600 | 73.6 | 0.32 | 33,200 |
| Treated with rivanol and removed the rivanol in the solution. | 12,320 | 67,300 | 42.9 | 5.47 | 11,950 |
| Adsorbed on resin at pH 5.0 and eluted with 1.0 *M* potassium hydroxide. | 2,440 | 32,500 | 20.8 | 29.7 | 1,000 |
| Fractionated with ammonium sulfate (40–60% sat.). | 104 | 22,750 | 14.5 | 220.0 | 500 |
| Adsorbed on resin at pH 5.5 and impurities removed with 0.3 *M* acetate buffer. | 36 | 18,890 | 12.1 | 530.0 | — |
| Impurities removed with resin at pH 6.5. | 14 | 16,733 | 10.6 | 1,190.0 | — |

* Primary digests with the ε-lysine acylase contained 2.5 ml. of 0.02 *M* $N^{\varepsilon}$-benzoyl-DL-lysine plus 2.5 ml. of 0.02 *M* phosphate buffer at pH 6.0 containing the enzyme and the digests with the α-lysine acylase contained 2.5 ml. of 0.02 *M* $N^{\alpha}$, $N^{\varepsilon}$-dibenzoyl-DL-lysine plus 2.5 ml. of 0.02 *M* phosphate buffer at pH 7.8 containing the enzyme.

taining the active protein, with 0.4 volume of saturated ammonium sulfate solution, the purified enzyme was precipitated almost quantitatively by 0.6 volume of the sulfate solution. The precipitate was centrifuged, redissolved in cold water, and dialyzed for 24 hours against running water. The enzyme was again adsorbed on 30 ml. of Amberlite XE-64 at pH 5.5. To remove the accompanying proteins, the resin was washed with a 0.3 *M* acetate buffer at pH 5.5, and the enzyme was eluted with 50 ml. of a 1.0 *M* acetate buffer at pH 5.5. The eluate was passed through a column of Amberlite XE-64 buffered with 0.02 *M* phosphate buffer at pH 6.5 so as to remove impurities. Lyophilization of the resulting solution yielded a nearly white preparation having a specific activity against $N^{\varepsilon}$-benzoyl-L-lysine of 1190 $\mu$ moles/hour/mg. of protein, representing about 4,000-fold purification of the activity in the original extract. Table I summarizes the specific activities and yields attained at the various steps of the purification procedure.

### *Properties of the ε-Lysine Acylase of KT-83*

*Influence of pH and Temperature on α- and ε-Lysine Acylase Activity*—The effect of pH at a constant phosphate concentration is illustrated in Fig. 1.

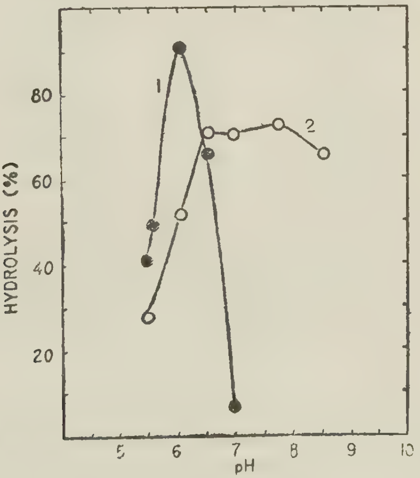


**FIG. 1.** pH-Activity curve of the highly purified ε-lysine acylase (Curve 1) and the crude α-lysine acylase (Curve 2). Hydrolysis of $N^{\varepsilon}$-benzoyl-L-lysine by the ε-lysine acylase and that of $N^{\alpha}$, $N^{\varepsilon}$-dibenzoyl-L-lysine by the α-lysine acylase. Digest consisted of 2.5 ml. of 0.02 *M* substrate plus 2.5 ml. of 0.02 *M* phosphate buffer at various pH's containing 0.1 mg. of the ε-acylase or 30 mg. of the α-acylase; incubation time, 1 hour.

The crude α-lysine acylase has an optimum pH at 7.8; the highly purified ε-lysine acylase at 6.0. The optimum temperature of both the α- and ε-lysine acylase is 37–38°. In further experimentation, the enzymic digestion was conducted under these conditions.

*Effect of Various Ions on Enzymic Action*—Studies of the effect of various metals on the activity of the purified enzyme were complicated in many cases by formation of insoluble phosphate salts. Fe$^{++}$, Ni$^{++}$, Ca$^{++}$, Mn$^{++}$, Co$^{++}$, Mg$^{++}$, Zn$^{++}$ and Fe$^{+++}$ in the form of sulfate or chloride were investigated at $10^{-3}\,M$ final concentration in citrate buffer at pH 6.0. Table II summarizes the effect of various ions on the enzymic action. At the present state of purification the enzyme was strongly activated by Zn$^{++}$ at $10^{-3}\,M$ concentration, the hydrolysis of $N^{\epsilon}$-benzoyl-DL-lysine being increased by 209 per cent (Table III). The $\alpha$-acylase (acylase I) was activated to a comparable extent by Co$^{++}$ (*9*).

TABLE II

*Effect of Various Ions on Enzymic Action*

| Metal ion ($10^{-3}\,M$) | Hydrolysis of $N^{\epsilon}$-benzoyl-L-lysine* (%) |
|---|---|
| Fe$^{++}$ | 20.0 |
| Fe$^{+++}$ | 23.6 |
| Ni$^{++}$ | 24.6 |
| Ca$^{++}$ | 22.6 |
| Mn$^{++}$ | 27.6 |
| Mg$^{++}$ | 24.6 |
| Co$^{++}$ | 25.6 |
| Zn$^{++}$ | 41.4 |
| EDTA | 31.5 |
| None | 19.8 |

* Primary digests contained 2.5 ml. of $0.02\,M$ $N^{\epsilon}$-benzoyl-DL-lysine plus 2.5 ml. of $0.1\,M$ citrate buffer at pH 6.0 containing various ions and 0.05 mg. of the purified enzyme; incubation time, 1 hour.

TABLE III

*Degree of Activation and Zn$^{++}$ Concentration at 0.01 M $N^{\epsilon}$-Benzoyl-DL-lysine*

| | Zn$^{++}$ at concentration of | | | | | |
|---|---|---|---|---|---|---|
| | 0 | $1\times10^{-4}\,M$ | $2\times10^{-4}\,M$ | $1\times10^{-3}\,M$ | $2\times10^{-3}\,M$ | $1\times10^{-2}\,M$ |
| Hydrolysis of $N^{\epsilon}$-benzoyl-L-lysine* (%) | 16.6 | 27.5 | 30.6 | 34.8 | 34.8 | 34.6 |

* Primary digests contained 2.5 ml. of $0.02\,M$ $N^{\epsilon}$-benzoyl-DL-lysine plus 2.5 ml. of $0.1\,M$ citrate buffer at pH 6.0 containing various amounts of the Zn$^{++}$ and 0.05 mg. of the purified enzyme; incubation time, 1 hour.

The observations made by Wright and his co-workers (*10*) on the activation of biocytinase by various biologically occurring reducing agents indicated that similar conditions might obtain in the present system. Cysteine, ascorbic acid and potassium cyanide in concentrations up to 5 mg./ml. were found to increase slightly the rate of the asymmetrical hydrolysis of $N^{\varepsilon}$-benzoyl-DL-lysine by the enzyme.

*Electrophoresis Studies*—From the results of paper electrophoresis, using the veronal buffer at pH 8.8, $\mu$ 0.05, for one hour, it was found that the purified enzyme obtained by the above procedures contained still two protein fractions, though the enzyme no longer showed $\alpha$-lysine acylase activity and its specific activity was about 4,000 times as high as that of the crude material. One of the proteins moved to the cathode; the other to the anode. The former possessed the activity of $\varepsilon$-lysine acylase while the latter showed no such activity (Fig. 2).

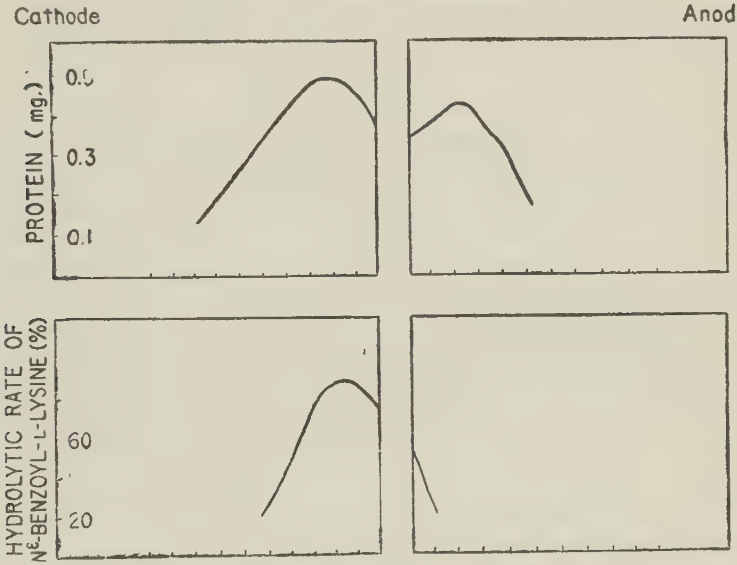


Fig. 2. Electrophoresis of the $\varepsilon$-lysine acylase and its activity. Paper electrophoresis of the purified enzyme was conducted using veronal buffer at pH 8.8, $\mu$0.05, in one hour. Each piece of paper, prepared by cutting the strip at 3-mm. intervals, was extracted with 2 ml. of 0.02 *M* phosphate buffer at pH 6.0 and examined.

*Specificity*—Table IV lists the relative activities of the enzyme preparation against acetylated and benzoylated lysine. The hydrolysis of diacylated lysines by the enzyme was examined with ninhydrin reagent and the recovery of the starting material in the incubated solution using organic solvents and electrophoresis. The enzyme does not attack $N^{\alpha}$, $N^{\varepsilon}$-diacyl derivatives of lysine; nor does it hydrolyze $N^{\varepsilon}$-acyl-D-lysine derivatives. $N^{\varepsilon}$-acyl-L-lysine derivatives are hydrolyzed by the enzyme and the benzoyl derivative is cleaved at a more rapid rate than the acetyl.

TABLE IV

*Hydrolysis of Acylated* L-*Lysine*

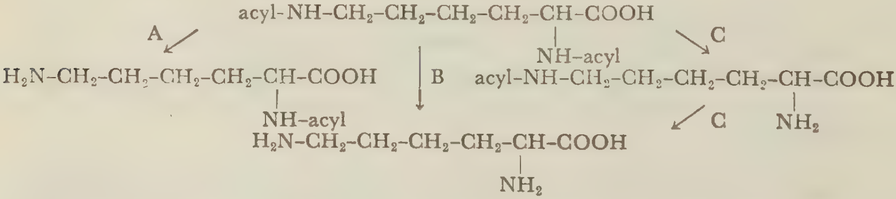
| Substrate | Extent of hydrolysis[1] (%)[2] |
|---|---|
| $N^{\alpha}$, $N^{\varepsilon}$-dibenzoyl-DL-lysine | — |
| $N^{\varepsilon}$-benzoyl-DL-lysine | 75.0 |
| $N^{\alpha}$, $N^{\varepsilon}$-diacetyl-DL-lysine | — |
| $N^{\varepsilon}$-acetyl-L-lysine | 21.6 |
| $N^{\varepsilon}$-acetyl-DL-lysine | 30.6 |

1) Primary digests contained 2.5 ml. of 0.02 *M* acylated lysine plus 2.5 ml. of 0.02 *M* phosphate buffer at pH 6.0 containing 0.07 mg. of the purified enzyme; incubation time, 1 hour.

2) Based on the hydrolysis of the L-form. The D-form remains unchanged.

## DISCUSSION

Kameda and his collaborators* succeeded in obtaining L-lysine and $N^{\alpha}$, $N^{\varepsilon}$-dibenzoyl-D-lysine from $N^{\alpha}$, $N^{\varepsilon}$-dibenzoyl-DL-lysine by the action of the cells of KT-83, their acetone powder and their cell free enzyme. The present author also obtained L-lysine and $N^{\alpha}$, $N^{\varepsilon}$-diacetyl-D-lysine from $N^{\alpha}$, $N^{\varepsilon}$-diacetyl-DL-lysine by the action of the above enzymes. With respect to these asymmetrical hydrolysis of $N^{\alpha}$, $N^{\varepsilon}$-diacyl-DL-lysine, the following three possible pathways are considered. Pathway (A): by the action of the ε-lysine acylase of KT-83, $N^{\alpha}$, $N^{\varepsilon}$-diacyl-DL-lysine undergoes asymmetrical hydrolysis to give $N^{\alpha}$-acyl-L-lysine, which is then hydrolyzed to L-lysine by the α-lysine acylase. Pathway (B): by the independent action of the α-lysine acylase and the ε-lysine acylase, $N^{\alpha}$, $N^{\varepsilon}$-diacyl-DL-lysine is asymmetrically hydrolyzed to L-lysine. Pathway (C): $N^{\alpha}$, $N^{\varepsilon}$-diacyl-DL-lysine is first hydrolyzed asymmetrically by the α-lysine acylase to $N^{\varepsilon}$-acyl-L-lysine, which is then hydrolyzed to L-lysine by the ε-lysine acylase.


acyl-NH-CH2-CH2-CH2-CH2-CH-COOH
NH-acyl
A
C
H2N-CH2-CH2-CH2-CH2-CH-COOH
NH-acyl
B
acyl-NH-CH2-CH2-CH2-CH2-CH-COOH
NH2
C
H2N-CH2-CH2-CH2-CH2-CH-COOH
NH2

The ε-lysine acylase which does not possess the activity of the α-lysine acylase, is unable to hydrolyze $N^{\alpha}$, $N^{\varepsilon}$-diacyl-DL-lysine, that is to say, it does not hydrolyze the acyl group at ε-position of $N^{\alpha}$, $N^{\varepsilon}$-diacyl-DL-lysine, though it asymmetrically hydrolyzes $N^{\varepsilon}$-acyl-DL-lysine easily. On the other hand, the α-lysine acylase of KT-83 asymmetrically hydrolyzes the acyl group at α-position of $N^{\alpha}$, $N^{\varepsilon}$-diacyl-DL-lysine very easily.

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* Private communication from Dr. Y. Kameda, Kanazawa University.

From these facts, it is concluded that the asymmetrical hydrolysis of $N^\alpha$, $N^\varepsilon$-diacyl-DL-lysine by the acylases of KT-83 should follow the pathway (C), that is to say, $N^\alpha$, $N^\varepsilon$-diacyl-DL-lysine is asymmetrically hydrolyzed by the $\alpha$-lysine acylase to $N^\varepsilon$-acyl-L-lysine, which is then hydrolyzed to L-lysine by the $\varepsilon$-lysine acylase.

## SUMMARY

Three different kinds of acylases, $\alpha$-lysine acylase, $\varepsilon$-lysine acylase and D-phenylalanine acylase, capable of performing the resolution of acyl-DL-amino acids, are produced by a *Pseudomonas* species, KT-83, which was previously isolated from soil by Kameda *et al.* One of these enzymes, $\varepsilon$-lysine acylase, was purified by treatment with ion exchange resin and by fractionation with ammonium sulfate. The purified enzyme does not possess the activity of the $\alpha$-lysine acylase, but it has a specific activity against $N^\varepsilon$-benzoyl-L-lysine, representing about 4,000-fold purification of the activity in the original extract. The $\alpha$-lysine acylase has an optimum pH at 7.8 and the $\varepsilon$-lysine acylase at 6.0. The optimum temperature of both the $\alpha$- and $\varepsilon$-lysine acylase is 37–38°. Among a variety of ions $Zn^{++}$ at $10^{-3}\,M$ concentration increases the activity of the $\varepsilon$-lysine acylase against $N^\varepsilon$-benzoyl-DL-lysine. The purified enzyme was shown by electrophoresis to contain two protein fractions.

The enzyme hydrolyzes asymmetrically $N^\varepsilon$-acyl-DL-lysine easily, but is unable to hydrolyze $N^\alpha$, $N^\varepsilon$-diacyl-DL-lysine. From these facts, the asymmetrical hydrolysis of $N^\alpha$, $N^\varepsilon$-diacyl-DL-lysine by the crude acylase preparation of KT-83 is considered to proceed as follows: $N^\alpha$, $N^\varepsilon$-diacyl-DL-lysine is first hydrolyzed asymmetrically by the $\alpha$-lysine acylase to $N^\varepsilon$-acyl-L-lysine, which is then hydrolyzed to L-lysine by the $\varepsilon$-lysine acylase.

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Thanks are also due to Dr. E. Ohmura, Mr. A. Miyake and Dr. K. Ogata for their support, and to Mr. I. Imada and Mr. K. Tomoda for their technical help.

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# ACTION OF INHIBITORS ON PYRUVIC OXIDASE, COCARBOXYLASE, AND THIAMINE

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A number of hypotheses have been proposed (*1–8*, *11*) on the mechanism of action and the nature of the active center of cocarboxylase, but the problems relating to the complex formation between cocarboxylase and substrate have not yet been worked out. In the present study, essential group responsible for the action of cocarboxylase were investigated by using various inhibitors in pyruvic oxidase system and a series of model experiments were carried out with thiamine and its derivatives.

## METHODS

*Pyruvic Oxidase System*—Pyruvic oxidase was purified from a dog heart homogenate by freezing and thawing according to the method of Jagannathan and Schweet (*8*). The assay system contained 100 $\mu$M of lithium pyruvate, 10 $\mu$M of magnesium chloride or manganese chloride, 50 $\mu$M of sodium bicarbonate, 10 $\mu$M of cysteine, and 0.4 $\mu$M of TDP in 0.8 ml. of aqueous solution. The solution, after being saturated with carbon dioxide, was placed in the main compartment of the Warburg flask and 1.5–2.0 ml. of the enzyme solution was added. 100 $\mu$M of potassium ferricyanide as an electron acceptor and 0.3 ml. each of various inhibitors such as PCMB and IAA was placed in the side arm and tipped.

*System for the Acetoin Formation Catalyzed by Thiamine*—According to the method described by S. Mizuhara (*6*) 0.5 ml. of 0.1 *M* thiamine, 0.1 ml. of 1 *N* NaOH, 1.0 ml. of 0.1 *M* lithium pyruvate, 0.2 ml. of 1 *M* acetaldehyde were dissolved in 4.0 ml. of water, the final pH being 8.4. After being evacuated in a Thunberg tube, the mixture was incubated at 37°. The system contained two equivalents of alkali to thiamine.

*Paper Partition Chromatography*—Solvent I: Pyridine-isobutanol-water (2 : 3 : 5); for the separation of thiamine, TDP, thiochrome, PCMB, and thiamine combined with PCMB. Solvent II: Butanol-ethanol (4 : 1) saturated with 2 *N* ammonium carbonate, for PCMB. Solvent III: Butanol-acetic acid-water (4 : 1 : 5), for thiamine and TDP. The materials were applied to 'Toyo' filter paper No. 3 or No. 50.

Cyclic compounds such as pyrimidine, PCMB or thiamine were detected on paper chromatogram by ultraviolet rays (Fig. 2) and thiochrome by its fluorescence when irradiated with ultraviolet rays. Thiamine was also located by thiochrome reaction. PCMB was detected by spraying diphenylcarbazone after heating the paper at 100° for 1 minute and spraying with $N/30$ $AgNO_3$. The carboxyl group of PCMB was detected as a yellow spot of ammonium salt after spraying methyl red.

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* Present address: Department of Biochemistry, School of Medicine, Juntendo University, Tokyo. The following abbreviations are used: monoiodoacetate, IAA; *p*-chloromercuribenzoate, PCMB; thiamine diphosphate, TDP.

*Determination of Acetoin*—The formation of acetoin was determined according to the method of Westerfeld (*10*). However, as IAA interfered with the coloration by releasing iodine which was mixed with the distillate of diacetyl, experiment with IAA could not be performed to observe the inhibition of acetoin formation catalyzed by thiamine. Other substances which were present in the reaction system did not interfere with coloration reactions.

*TDP Thiamine, and Its Derivatives*—These authentic substances were synthesized in the Research Laboratory attached to Takeda Pharmaceutical Industries and were generously donated by Dr. T. Matsukawa.

## RESULTS

*Effect of Inhibitors on Pyruvic Oxidase System*—In the assay system 10 $\mu$M of IAA ($3.3\times10^{-3}$ *M*), 0.3 $\mu$M PCMB ($10^{-4}$ *M*), or 0.05 $\mu$M of furacin ($1.7\times10^{-5}$ *M*) was added, but no decrease in the production of carbon dioxide was observed (Fig. 1). This fact indicates that the enzyme-substrate complex is so stable that these inhibitors can not combine with the enzyme or TDP by excluding the substrate. The following experiments will further confirm the above interpretation.

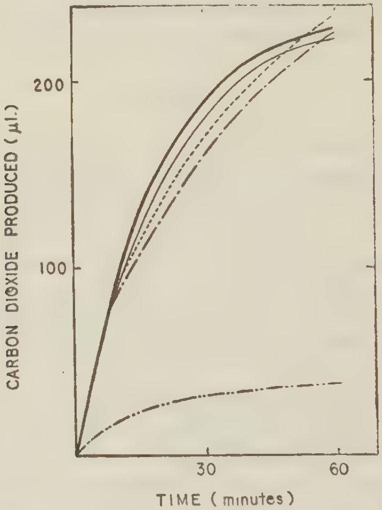


FIG. 1. Effect of inhibitors on pyruvic oxidase system.
—— standard assay system, —— with furacin ($1.7\times10^{-5}$ *M*), ---- with IAA ($3.3\times10^{-3}$ *M*), —·—·— with PCMB ($10^{-4}$ *M*), —··—··— without TDP.

With components of the assay system, the same as described above, the order of adding the components was altered as follows: the enzyme solution was first incubated with one of the inhibitors such as IAA, PCMB (the same concentration described as above), and $K_3Fe(CN)_6$ (100 μM) in the buffer solution for 30 minutes at 37°, and the substrate, coenzyme, and an electron acceptor, $K_3Fe(CN)_6$, were then added. The markedly decreased production of carbon dioxide by the SH-blocking agents (Table I) showed that the -SH of apoenzyme is one of the functional groups for the formation of enzyme-substrate complex.

### TABLE I

*Effect of Inhibitors on Apoenzyme of Pyruvic Oxidase*

| Inhibitors | No addition of inhibitors | Inhibitors added | | |
|---|---|---|---|---|
| | | IAA (10 μM) | PCMB (0.3 μM) | $K_3Fe(CN)_6$ (100 μM) |
| $CO_2$ produced during 30 minutes (μM) | 5.8 | 1.25 | 2.8 | 1.16 |

A solution containing 50 μM of bicarbonate, 10 μM of magnesium chloride, and an inhibitor was saturated with carbon dioxide and placed in the main compartment of a Warburg flask, 1.5 ml. of the enzyme solution was added, and incubated at 37°. Then, a solution of 100 μM of lithium pyruvate, 10 μM of cysteine, 0.4 μM of TDP, and 100 μM of potassium ferricyanide was tipped from the sidearm.

### TABLE II

*Effect of Inhibitors on Cocarboxylase*

| Addition of TDP | − | + | − | − | − | + |
|---|---|---|---|---|---|---|
| Inhibitors | − | − | − | IAA<br>+ | PCMB<br>+ | Reinecke salt<br>+ |
| TDP* treated with diluted alkali | − | − | + | + | + | − |
| $CO_2$ produced during 1 hour (μM) | 2.3 | 9.1 | 8.8 | 3.6 | 5.5 | 6.6 |

* The solution containing TDP and inhibitor was adjusted to pH 10 and kept at 0°. After 30 minutes it was neutralized with hydrochloric acid. Other conditions were the same as described in Table I.

*Effect of Inhibitors on Cocarboxylase*—Each inhibitor was added to TDP solution at 0° and pH of the solutions was adjusted to 10 with potassium hydroxide. The solutions were kept at 0° for 30 minutes to form mainly the thiol type (*3*) of TDP and to combine the inhibitors with this type. After pH of the solutions was readjusted to 6.5 with hydrochloric acid, the solutions were used for pyruvic oxidase system to compare with the standard assay system with non-treated TDP. The results are shown in Table II. From the decreased formation of carbon dioxide when the SH-blocking agents or Reinecke salt which blocks quaternary ammonium base is added, it may be considered that the active center of the cocarboxylase is not only SH group formed by opening of the thiazole ring at pH 10, but also the original base. This point will be discussed in more detail later.

*Inhibition of Acetoin Formation by PCMB in Model Experiments*—When 0.75 ml. of 0.02 *M* solution of PCMB was added to the complete system of acetoin formation catalyzed by thiamine, the acetoin formation decreased to 20 per cent of that of the complete system (Table IV). *O,S*-Diacetylthiamine (Table III) can catalyze the acetoin formation as does thiamine (Table IV). In this case PCMB inhibits the reaction presumably by combining with the thiol group which may be formed by hydrolysis of -S-CO- bond that occurs in alkaline solution at 37°, as Matsukawa has reported (*11*).

TABLE III

*Chemical Structures of the Thiamine Derivatives Used*

| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| *O,S*-Diacetylthiamine | $-COCH_3$ | $-COCH_3$ | $-NH_2$ |
| *O*-Benzoyl-*S*-carboxymethylthiamine | $-CH_2COOH$ | $-COC_6H_5$ | $-NH_2$ |
| Oxythiamine | — | $-H$ | $-OH$ |

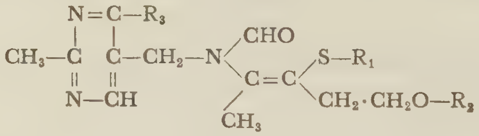


TABLE IV

*Formation of Acetoin Catalyzed by O,S-Diacetylthiamine*

| Catalyst | | *M*/10 Thiamine | | *M*/10 Diacetylthiamine | |
|---|---|---|---|---|---|
| *M*/50 PCMB (0.75 ml.) | | − | + | − | + |
| Acetoin formation (µg.) | 6 hrs. | 118 | 24.2 | 89.0 | 11.2 |
| | 18 hrs. | 322 | | 313 | 33.6 |

It was found that a remarkable decrease of acetoin formation occurred when oxythiamine (Table III) was used instead of thiamine (Table V), and the inhibitory action of PCMB was also observed in this reaction.

*Detection of Thiamine Combined with PCMB*—The solution of the complete system for acetoin formation catalyzed by thiamine which contained PCMB was analyzed by paper chromatography (Fig. 2). Thiamine, thiochrome and PCMB were located by the method described above. When the paper was sprayed with 0.4 per cent cysteine solution and heated at 70° for 15 minutes (*13*), two spots showing thiochrome reaction appeared at the position of $R_f$ 0.49±0.02 and $R_f$ 0.73−0.74 (Solvent I). Further, the spots showed the same reaction for mercury of PCMB with diphenylcarbazone method.

TABLE V

*Formation of Acetoin with Oxythiamine*

| | | | |
|---|---|---|---|
| $M/10$ Thiamine | + | − | − |
| $M/10$ Oxythiamine | − | + | + |
| $M/50$ PCMB (0.75 *ml.*) | − | − | + |
| Acetoin formation during 18 hours (*μg.*) | 322 | 74 | 25 |

The band, $R_f$ 0.49 of this paper chromatogram was eluted with distilled water (pH was adjusted to 7.0 with potassium hydroxide) and the solution was concentrated under a reduced pressure in carbon dioxide stream to avoid formation of thiochrome or thiothiamine. About 4 mg. of faint yellow-white powder which decomposed at 177–182° was obtained as a residue.

This substance X was rechromatographed with solvent I and appeared as one spot at $R_f$ 0.53 by diphenylcarbazone method, or thiochrome reaction after spraying 0.4 per cent cysteine solution, and heating. After spraying potassium cyanide, the spot was detected with Grote's reagent and showed a faint blue color which suggests the presence of thiol group. This reaction could not be found at the spot of thiamine. The substance X is slightly soluble in distilled water and acid solution, differing from thiamine disulfide. The solution of X showed thiochrome reaction with potassium ferricyanide and alkali only after being reduced with hydrogen sulfide. Two milligrams

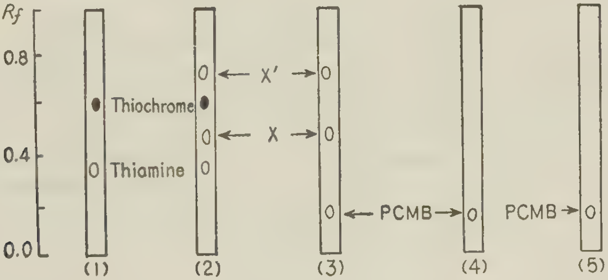


FIG. 2. Analysis of thiamine-PCMB compound by paper chromatography.
● represents the spot showing fluorescence under ultraviolet rays.

(1), sprayed with the reagents for thiochrome reaction. (2), 0.4 per cent solution of cysteine was sprayed and after being heated, localized by thiochrome reaction. (3), (4), reaction for mercury. (5), yellow spot after spraying methyl red. Solvent I: for (1), (2), (3). Solvent II: for (4), (5). See text for detail.

of X was dissolved in water of pH 7.5, 2 $N$ hydrochloric acid was added, and the solution was heated for 10 minutes at 60°. The white precipitate produced was identified as PCMB by ultraviolet absorption curve ($\lambda_{max.}$= 235 m$\mu$) (Fig. 3) and by paper chromatographic method (Fig. 2). From the

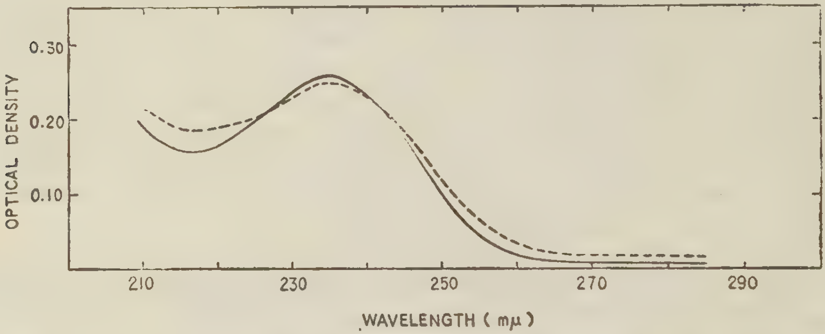
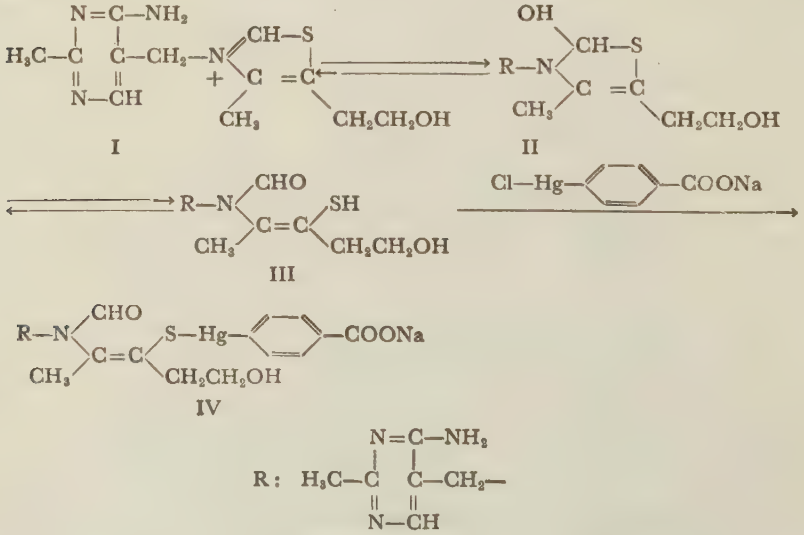


FIG. 3. Ultraviolet absorption curves of PCMB and decomposition product of substance X.
—— PCMB (authentic), - - - material eluted from paper chromatogram.

### DIAGRAM I

Formation of thiol type of thiamine and its binding with PCMB (in pH-8 4 solution at 37°).



supernatant the presence of thiamine was confirmed by thiochrome reaction of paper chromatogram. The solution of X produced nitrogen more promptly than in the case of thiamine solution when iodine-azide test was carried out.

The crystals of thiochrome and 2-methyl-4-amino-5-aminomethylpyrimidine were each incubated with PCMB in a solution of pH 8.4 for 18 hours at 37°, but no evidence that PCMB combined with any functional group such as $-OH$ of thiochrome or $-NH_2$ of pyrimidine could be obtained by paper chromatography (detected with absorption of ultraviolet rays and diazo reaction).

From these analyses it may be concluded that PCMB combines with the thiol type of thiamine forming a mercaptide (Fig. 4, IV), but not with thiochrome or the pyrimidine moiety of thiamine.

Substance X, in the concentration of about *M*/100, could not catalyze the acetoin formation from pyruvate and acetaldehyde, while thiamine solution of the same concentration could do it.

It was found that substance X′, which showed $R_f$ 0.73 on the paper chromatogram, had the same properties as X in the eluted solution from paper chromatogram except that it was soluble in acid solution and PCMB could not yet be separated.

*O-Benzoyl-S-carboxymethylthiamine as Catalyst*—Monoiodoacetate could not be used in the above experiments as an inhibitor of acetoin formation because it interfered with determination of acetoin, but *O*-benzoyl-*S*-carboxymethylthiamine (Table III) had only a little effect as a catalyst for the acetoin formation (Table VI). As this substance is a thiamine derivative, thiol group of which has reacted with monoiodoacetate, the thiol group must be essential for the catalytic action.

TABLE VI

*Formation of Acetoin with O-Benzoyl-S-carboxymethylthiamine*

| Catalyst | *M*/20 Thiamine | *M*/20 *O*-Benzoyl-*S*-carboxymethylthiamine |
|---|---|---|
| Acetoin formation during 18 hours (*μg.*) | 137 | 29.0 |

## DISCUSSION

Previously, Langenbeck (*1*) proposed on the action mechanism of TDP the hypothesis of the formation of a Schiff base between carbonyl group of pyruvic acid and amino group of pyrimidine of TDP. The formation of hydrogen bond between those groups was suggested by Singer and Pensky (*5*). On the other hand, many workers have supported the theory of quaternary ammonium base of thiazole as the active center of cocarboxylase. Lately, Mizuhara (*6*) found the catalytic action of thiamine in the formation of acetoin from pyruvic acid and acetaldehyde without an apoenzyme, and according to his experiments it is considered that the tertiary nitrogen of thiamine pseudobase is the active center for the reaction.

From the results obtained in the present series of experiments it may be concluded that the SH-blocking agents inhibit those catalytic actions, by reacting with thiol group of apoenzyme or thiol type of TDP as the theory proposed by Matsukawa (*11*). The fact that TDP is labile in alkaline solution made the present experiments difficult. Utilizing the model experiments of thiamine, however, the action of TDP was presumed and a reaction was proved to take place between PCMB and the thiol group of thiamine. According to polarographic studies (*12*), there are some evidence that thiamine can form its thiol type already in neutral solution.

Moreover, it is apparent that the amino group of thiamine is essential to acetoin formation, because of the remarkable decrease of its formation with oxythiamine*.

*O*, *S*-Diacetylthiamine can catalyze the acetoin formation as much as thiamine does. This indicates the possibility that the tertiary nitrogen of thiazole takes part in its catalytic action. The effect of Reinecke salt on cocarboxylase also supports this possibility, but if the thiol group is to be freed by hydrolysis –S–CO– bond in diacetylthiamine in alkaline solution, it can not be denied that the thiol group is one of the active centers, which can also be deduced from the experiment with *O*-benzoyl-*S*-carboxymethylthiamine.

Accordingly it may be concluded that thiamine possibly participates in acetoin formation with all of its functional groups such as thiol group, tertiary nitrogen of thiazole, and amino group of pyrimidine, and this conclusion might be applicable to the action of TDP.

## SUMMARY

1. The action of various inhibitors on pyruvic oxidase system, cocarboxylase, or thiamine was examined. The catalytic action of thiamine derivatives was also studied instead of thiamine in the reaction of acetoin formation.

2. Thiamine combined with PCMB was detected and analyzed. It could be concluded that PCMB combines with the thiol group of thiamine.

3. From the experimental results the active center of thiamine or TDP was discussed. It was concluded that –SH, $-NH_2$, and thiazole-N were possibly responsible for their catalytic action.

The author wishes to express his gratitude to Prof. N. Shimazono and Dr. N. Hosoya for their encouragement and helpful suggestions.

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* These reports were presented at the 28th Meeting of the Japanese Biochemical Society on November 1, 1955. Breslow (*14*) also reported that oxythiamine could not catalyze acyloin condensation in the non-enzymatic system described by Mizuhara.

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# THE ROLE OF CALCIUM IN TAKA-AMYLASE A

## II. THE EXCHANGE REACTION OF CALCIUM

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Although Taka-amylase A (TAA) crystallized in the presence of calcium salts contains about 10 atoms of calcium per mole of protein, most of them do not seem to be essential for the enzymatic activity. Thus, when such TAA was dialysed against sodium acetate solution, 9 out of the 10 calcium atoms are readily removed from the protein without loss of enzymatic activity. The last one atom of calcium which resists to the dialysis seems to be rather firmly bound to the protein and can be detached only in the presence of ethylenediamine tetraacetic acid (EDTA). It is therefore probable that this calcium atom is an integral part of the TAA molecule. Furthermore, it was found that the removal of the last calcium atom from TAA in the presence of EDTA resulted in the inactivation of the enzyme and a simultaneous increase in levo-rotatory power of the solution. These findings were in accord with the view that this calcium atom is essential for the maintenance of the catalytically active structure of TAA (*1*).

The present paper describes the results of an investigation which was undertaken to obtain further information concerning the indispensability of the calcium atom to the maintenance of active structure, its affinity to the protein, and the mechanism by which it plays its essential role. The mechanism of acid inactivation of TAA (*2*) is also discussed based on the results obtained in the present investigation.

### EXPERIMENTALS

### *Materials and Methods*

*Normal TAA*—Crystalline "normal" TAA was prepared from "Takadiastase Sankyo" by the method of Akabori *et al.* (*3*) and recrystallized 3 or 4 times from aqueous acetone.

*TAA Crystallized from Abnormal Culture Fluids*—*Aspergillus oryzae* was grown on the surface of a medium containing strontium instead of calcium (Sr culture) and of another medium containing no added calcium (Ca-free culture) at 29° for 19 days. The mycelial pads were then removed from the both cultures and TAA was isolated in crystalline states according to the usual procedures except that strontium acetate solution was used instead of calcium acetate solution.

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The composition of "Ca-free medium" was as follows: Glucose, 50 g.; maltose, 10 g.; potassium chloride, 1.5 g.; disodium phosphate, 17.9 g.; malonic acid, 2.6. g.; ammonium tartrate, 9.2 g.; magnesium sulfate, 0.24 g.; ferrous sulfate, 3 mg.; distilled water, 1,000 ml.; pH adjusted to 5.5 (*cf.* (*4*)). 0.55 g. of strontium chloride (4 mM) was added to each 1 liter of Ca-free medium to prepare "Sr medium". All the chemicals employed were either analytical or first grade reagents and used without further purification. The use of starch as the carbon source was avoided because of its high calcium contents.

*Ca$^{45}$-labeled TAA*—1.5 g. of normal (unlabeled) TAA were washed 3 times with 55 per cent aqueous acetone, dissolved in distilled water, and freed from acetone by dialysing the solution over night against a dilute calcium acetate solution. The acetone-free solution was further dialysed at 5° for 5 days against 0.02 *M* sodium acetate to remove the dialysable (non-essential) calcium atoms. To this solution were added about 15 mg. of Ca$^{45}$Cl$_2$ (20 $\mu$c./mg.) and the mixture was allowed to stand at 6° for 9 days. TAA was then precipitated from the solution with acetone, redissolved in 0.02 *M* calcium acetate and a small amount of denatured protein was removed by centrifugation. Ca$^{45}$-labeled TAA was finally crystallized with acetone from the clear supernatant.

Prior to use, the crystalline labeled TAA was dissolved in water and dialysed against 0.02 *M* sodium acetate in order to remove the dialysable calcium atoms. As shown in Table I, the specific radioactivity of TAA became constant after 4 days dialysis.

TABLE I

*Change of Radioactivity of Ca$^{45}$-labeled TAA during Dialysis*

| Expt. No. | Time of dialysis (*day*) | *c.p.m.* (corrected) | TAA (*mg.*) | Specific count (*c.p.m./mg.-TAA*) | |
|---|---|---|---|---|---|
| | | | | | Average |
| 1. | 0 | 3015<br>2075<br>3880 | 0.546<br>0.364<br>0.710 | 5520<br>5700<br>5470 | 5540 |
| | 5 | 387±10<br>358±10<br>281± 9 | 1.16<br>1.02<br>0.75 | 334± 9<br>353±10<br>377±12 | 355±34 |
| | 7 | 366± 6<br>349± 7<br>401± 7 | 1.02<br>0.95<br>1.14 | 361± 6<br>366± 7<br>351± 7 | 360±16 |
| 2. | 4 | 256± 6<br>168± 5<br>273± 6<br>99± 3 | 0.945<br>0.670<br>1.073<br>0.412 | 271± 6<br>251± 7<br>254± 6<br>240± 8 | 254±23 |
| | 7 | 427± 7<br>427± 7<br>273± 6<br>324± 6 | 1.758<br>1.675<br>0.883<br>1.30 | 243± 4<br>255± 4<br>268± 6<br>250± 5 | 254±20 |

*Measurements of Radioactivity*—The radioactivity of Ca$^{45}$-labeled TAA was measured with a Nuclear-Chicago D47 gas-flow counter with a "Micromil" window. The dry weight of each sample was in the range of 0.5 to 2 mg. per 2.5 square centimeters and hence no correction for self-absorption was necessary. The coincidence correction was also omitted since all the measured activities, with one exception (Table I), were less than 600 counts per minutes. Counting was made on 2 to 4 different aliquots from the same sample and the values obtained were averaged. For computation of specific activity, the dry weight of TAA on each sample pan was calculated from the following equation;

$$\text{dry weight of TAA} = w \cdot W_T / W,$$

where, w=dry weight of sample in the pan; $W_T$=TAA concentration (mg./ml.) in test solution as determined by the micro-Kjeldahl method (see below); and W=dry weight of 1 ml. of test solution (mg./ml.).

*Determination of Alkaline Earth Metals*—The TAA solution to be analysed was freed from the dialysable metals by dialysis against 0.02 *M* sodium acetate for 4 to 6 days with occasional change of the acetate solution. An aliquot of the dialysed solution was then ashed in a platinum crucible, a suitable amount of distilled hydrochloric acid was added to the ash and the mixture was dried. The dried residue was dissolved in 1 ml. of distilled water, and the solution was analysed by flame photometry for its contents of calcium, strontium and magnesium.

*Measurement of Enzymatic Activity*—The activity of TAA was measured by the Fuwa's method (*5*) and expressed in saccharifying power (S.P.) units.

*Measurement of Optical Rotation*—The optical rotations were determined with a Daika automatic mutarotation recorder Type 1 devised by Akabori and Tsugita and a Rudolf spectropolarimeter. Sodium and mercury lamps were used as light sources.

*Determination of TAA Concentration*—This was determined by the micro-Kjeldahl method. The concentration was calculated based on the fact that the nitrogen content of TAA is 14.98 per cent (*3*). In some experiments where limited amounts of sample had to be handled, the Folin's method was employed with a standard curve obtained with TAA solutions which had previously been determined by the micro-Kjeldahl method.

## RESULTS

*Exchangeability of Calcium in TAA*—In order to see if the essential (non-dialysable) calcium atom in TAA can exchange with exogenous calcium, $Ca^{45}$-labeled TAA which had been freed from dialysable calcium was mixed with non-labeled calcium acetate (100 moles per mole of TAA) and incubated at 20° for 2 days. The mixture was then dialysed against 0.02 *M* sodium acetate for 4 days with occasional change of the acetate solution. As can be seen from Table II (Expt. No. 1), it was shown by the radioactivity measurements before and after these treatments that 33 per cent non-dialysable calcium exchanged with exogenous calcium under these conditions.

A similar experiment (Table II, Expt. No. 2) was performed with non-labeled TAA to test the exchangeability of essential calcium with exogenous strontium. However, no strontium was detected in TAA after the contact of the enzyme with strontium followed by dialysis, a fact indicating that the affinity of calcium to the protein is much greater than that of strontium.

*Strontium Contents of TAA Obtained from Sr- and Ca-free Cultures*—Recrystallized preparations of TAA obtained from the Sr- and Ca-free cultures were dialysed against 0.02 *M* sodium acetate for 6 days, and their contents of calcium and strontium were determined. As shown in Table III, the both TAA preparations were found to contain 0.4 atom of calcium and 0.6 atom of strontium per mole of protein. The calcium in these preparations may have been derived from the impurities in the media. It is interesting to note that the sum of calcium and strontium is almost 1 atom per mole of TAA. Since the enzymatic activity of these strontium-containing preparations was the same with that of the normal TAA, it is suggested that strontium can replace calcium in keeping the enzyme molecule in the active structure.

TABLE II

*Exchange of Calcium in TAA*

| Expt. No. 1 | Ca to Ca | Specific count of TAA (*c.p.m./mg.-TAA*) | | Exchange (%) |
|---|---|---|---|---|
| | | Not treated | Treated | |
| | | 300±16 | 200±18 | 33 |
| Expt. No. 2 | Ca to Sr | Metal/TAA (*atom/mole*) found after treatment | | |
| | | Ca | Sr | |
| | | 0.73 | (−0.03) | 0 |

TABLE III

*Ca and Sr Contents in TAA Obtained from Sr- and Ca-free Cultures*

| TAA source | *atom/5 ml.* | | TAA conc. (*mg./ml.*) | *atom/mole-TAA* | | Enzymatic activity* (S.P./μg. at 37°) |
|---|---|---|---|---|---|---|
| | Ca | Sr | | Ca | Sr | |
| Ca-free-culture | 0.69 | 0.91 | 16.01 | 0.45 | 0.60 | 3.6 |
| Sr-culture | 0.36 | 0.53 | 9.46 | 0.40 | 0.59 | 3.5 |
| Taka-diastase | — | — | — | — | — | 3.4 |

* See the description under Table IV.

*Exchange of Ca with Mg, Sr and Ca during Crystallization Procedure*—Four times recrystallized normal TAA was dissolved in ice-cold 0.02 *M* magnesium acetate and then precipitated by the addition of cold acetone. The precipitate was again dissolved in the magnesium acetate solution and then precipitated. The precipitate was redissolved in 0.02 *M* magnesium acetate and allowed to stand for two days at room temperature, TAA was then precipitated. It could not be crystallized. The solution of this precipitate was centrifuged to remove small amounts of insoluble substance, and after 4 days' dialysis it was used for estimation of its metal content and enzymatic activity.

The treatment with strontium salt was carried out in just the same way. In this case the large granular crystals could be obtained. In the Ca-Ca exchange experiment, $Ca^{45}$-labeled TAA was treated in the same way. The results of these three experiments were summarized in Table IV.

TABLE IV

*Exchange of Metals in Crystallization Procedure*

| | Ca content | Mg or Sr content | Degree of exchange (%) | Enzymatic activity* (S. P./μg. at 37°) |
|---|---|---|---|---|
| | *(atom/mole-TAA)* | | | |
| Ca to Mg | 0.8 | 0.2 | 20 | 3.8 (3.2) |
| Ca to Sr | 0.42 | 0.30 | 40 | 4.8 (4.5) |
| | $Ca^{45}$ content *(c.p.m./mg.)* | | | |
| | Not treated | Treated | | |
| Ca to Ca | 294±16 | 156±13 | 47 | — |

* The enzymatic activity of TAA is affected by its preceding history, for example, time after dissolving into water, temperature at which TAA solution is stored. The storage at room temperature affects so TAA as to increase its activity (*6*). The different batch of substrate amylose gives different activity. The values shown in bracket are the value measured on normal TAA under similar conditions possible.

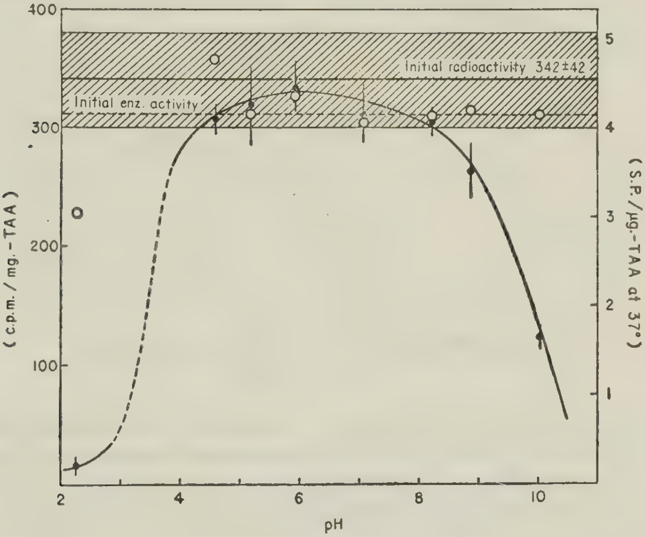

FIG. 1. Effect of pH on Ca exchange and enzymatic activity. Straight solid line is the level of specific radioactivity before treatment. Straight dotted line is the initial enzymatic activity. ● and ○ represent specific radioactivity and enzymatic activity after treatment.

*Exchange of Essential Calcium Atom at Various pH Values*—As described above, the essential calcium atom in $Ca^{45}$-labeled TAA is able to exchange with exogenous free calcium ions. It was therefore thought to be interesting to examine the effect of pH on the rate of the calcium exchange reaction.

One ml. of 3.08 per cent labeled TAA solution, which had been previously dialysed, was mixed with 4 ml. of 0.025 *M* calcium acetate solution (175 moles per mole of TAA) buffered at desired pH's (the pH's of the resulting mixtures were accurately determined). After standing for 1 hour at 17° they were neutralized to pH 7.2 and dialysed against 1 liter of 0.02 *M* sodium acetate for 4 days at 5°. Small amounts of insoluble matter formed during the dialysis were removed by centrifugation, and the clear supernatants were assayed for their radioactivity and enzymatic activity. As can be seen from Fig. 1, the specific radioactivity of TAA was only slightly decreased at the neutral pH region but greatly diminished at both acidic and alkaline regions. The enzymatic activity, on the other hand, was not depressed at all the pH values except at pH 2 where a considerable inactivation was observed.

*Relation between Acid Inactivation and Labilization of Calcium-Protein Linkage in TAA*—Since it was found that the essential calcium atom in TAA can exchange very rapidly with free calcium ions at pH 2, an experiment was designed to ascertain the parallelism between the enzymatic activity and the degree of labilization of calcium-protein linkage in TAA.

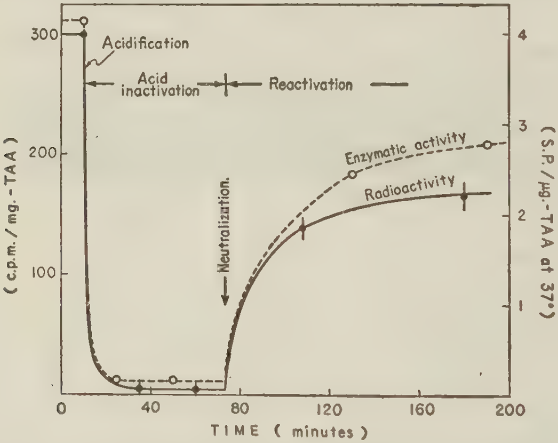

FIG. 2. Acid inactivation and the labilization of calcium-TAA linkage. Solid line is the specific count of TAA, which corresponds to the amount of TAA-bound calcium. Dotted line is the enzymatic activity, which was measured with the adequately diluted sample of an aliquot of TAA mixture. During the time elapsed from sampling to measurement (dilution and subsequent incubation in the presence of substrate) some restoration of the enzymatic activity must occurred.

A solution of $Ca^{45}$-labeled TAA, devoid of dialysable calcium atom, was acidified to pH 2.0 by adding phosphoric acid, and the mixture was allowed to stand at 21° for about 60 minutes. The solution was then rapidly neutralized to pH 6.8 with a pre-determined amount of sodium carbonate, and kept at the same temperature for another 120 minutes. During these treatments aliquots were withdrawn at suitable intervals and assayed for their enzymatic activity and the degree of calcium labilization. The latter was determined as follows. To the aliquot was added calcium acetate solution (*ca.* 100 moles per mole of TAA) and the mixture was immediately neutralized by adding a pre-determined amount of sodium carbonate to prevent further labilization of calcium-protein linkage, and then dialysed against 0.02 *M* sodium acetate for 4 days. That only a slight exchange takes place during dialysis has been shown in the preceding section. After the dialysis the specific radioactivity of the solution was determined. The aliquots taken from the reactivation stage (already neutralized!) were dialysed immediately after the addition of calcium acetate. In Fig. 2 are shown the results thus obtained. It is clearly shown that an excellent parallelism exists between the amount of non-exchangeable calcium and the enzymatic activity throughout the inactivation and reactivation stages.

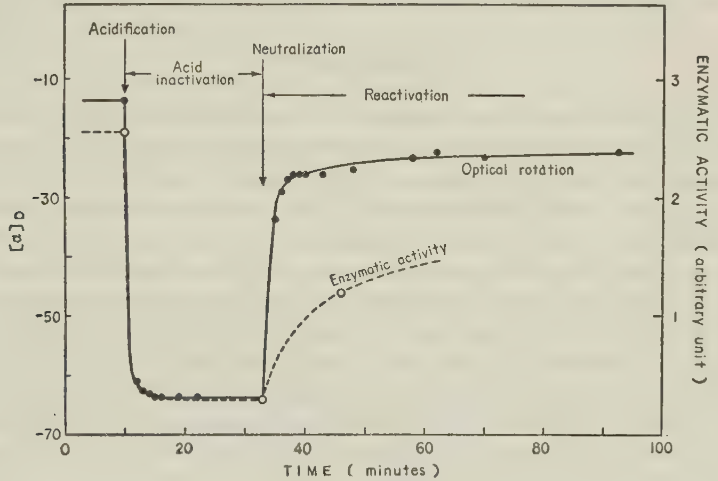

FIG. 3. Acid inactivation and $[\alpha]_D$ of TAA.

*Specific Optical Rotatory Power and Rotatory Dispersion of TAA during Acid Inactivation*—Dialyzed TAA was acidified by phosphoric acid to pH 2. After 23 minutes, the solution was neutralized by adding sodium carbonate. The levo-rotatory power of TAA showed a rapid increase with the progress of acid inactivation, and reached to a constant value. After neutralization, the levo-rotatory power reduced back with the restoration of enzymatic activity. Fig. 3 shows the time course of it. Then the rotatory dispersions of native, acid inactivated and reactivated TAA were measured for the purpose of searching the degree of unfolding. In the measurement after reactivation, $\lambda_c$ is regarded practically as constant during operation, because the $[\alpha]_D$ value before and after the measurements in various wave lengths was -21.2° and -20.0°, respectively, and it does not affect the calculation of $\lambda_c$. The results showed a simple dispersion obeying Drude's single term equation. The dispersion constants $\lambda_c$ were calculated from the modified equation introduced by Yang and Doty (*7*). The results were summarized in Table V.

TABLE V

*$[\alpha]_\lambda$ and $\lambda_c$ of TAA*

| Wave length ($m\mu$)<br>TAA | $[\alpha]_\lambda$ | | | | | $\lambda_c$ ($m\mu$) |
|---|---|---|---|---|---|---|
| | 365.02 | 404.66 | 435.83 | 546.07 | 58°.00 | |
| Native | − 58.0 | − 38.5 | − 29.0 | −14.4 | −12.0 | 280 |
| Acid inactivated[1] | −219. | −158.5 | −128. | −71.6 | −59.8 | 230 |
| Reactivated[2] | − 80.5 | − 57.0 | − 44.9 | −24.9 | −20.0<br>−21.2 | 245 |

1) The determination was carried out from 6 to 25 minutes after aciditication.

2) The determination was carried out from 23 to 45 minutes after neutralization.

## DISCUSSION

The fact that one atom of calcium (essential calcium) in the TAA molecule cannot be removed by dialysis against sodium acetate solution indicates that this calcium atom does not undergo an exchange reaction with the monovalent cation, sodium ion (*1*). In the present paper, however, it was clearly shown that the protein-bound calcium atom can readily exchange with exogenous free calcium ions (but not with strontium ions) under mild conditions. It was further found that the essential calcium atom can exchange even with strontium and magnesium ions when the enzyme was repeatedly recrystallized in the presence of these ions.

It is, however, quite reasonable to assume that calcium ion is the most adequate, among alkaline earth metal ions tested, to bind with the TAA protein, possibly because of its proper ionic radius. Thus, in aqueous solution, strontium cannot drive the protein-bound calcium atom out of the TAA molecule. In repeated recrystallization, however, it appears possible that even strontium or magnesium ions can enter into the TAA structure instead of calcium, probably due to some deformation which the enzyme protein suffered during the treatments. At any rate, such exchange reactions are easiest with calcium ion and most difficult with magnesium ion.

When TAA was isolated from culture fluids of *A. oryzae* grown in calcium-deficient media and purified in the presence of strontium acetate solution, the purified preparations of TAA were found to contain non-dialysable (firmly bound) strontium as could be expected. It was, however, shown that these preparations also contained non-dialysable calcium in addition to strontium, indicating that the trace quantities of calcium present in the media as impurities had been concentrated into the TAA molecule. This fact provides another evidence for a stronger affinity of calcium to the enzyme than of strontium. The question whether the non-dialysable strontium found in these preparations was built up into the protein during the production of TAA or during the purification procedures cannot be answered from the data presented in this paper.

Since it was shown that the TAA molecule containing either strontium or magnesium instead of calcium as its essential metal constituent is enzymatically as active as the normal calcium-containing TAA, it can be concluded that strontium and magnesium, if incorporated into the protein, can substitute the role of the essential calcium atom in maintaining the catalytically active structure of the protein.

TAA is rapidly inactivated under strongly acidic conditions (*2*), and TAA undergoes denaturation when the essential calcium is removed from the protein (*1*). The rapid exchange of calcium at acidic pH's observed in the present investigation seems to closely related to these facts. Under acidic conditions, the linkage between the essential calcium and the enzyme protein would be labilized and subsequently the active structure of TAA relaxed. This assumption seems to be verified by the experiments on the time course of labilization of calcium and enzymatic activity during acid inactivation and reactivation by neutralization. The experiments showed that the ratio of non-exchangeable calcium to the total essential calcium changed in parallel to the remaining or restored enzymatic activity. This suggests that the structure of TAA, in which calcium is deficient or at least weakly bound, is inactive and only potentially active.

The change in levo-rotatory power of TAA during the acid inactivation and reactivation processes seems to show that the so-called unfolding of helical structure of protein is going on in the inactivation process. But the possibility that the observed change in rotatory power is due to the change in electrical charge of TAA molecule, cannot be excluded. The fact that the reversal of $[\alpha]_D$ value is more rapid than the restoration of activity would also support the above possibility. The value of dispersion constant $\lambda_c$ during the inactivation and reactivation stages are suggestive of this problem. The $\lambda_c$ value of inactivated TAA falls into the range which most of denatured proteins exhibit (*7, 8*). The reversal of $\lambda_c$ during the reactivation process has not yet been measured since its measurement is very time-consuming. However, it is supposed from the limited results obtained that $\lambda_c$ would not reverse so rapidly as $[\alpha]_D$ did. If $\lambda_c$ would reflect the degree of unfolding more precisely than $[\alpha]_D$ at such an acidic pH, the activity seemed to depend very closely on the folded structure. For the complete elucidation of the relationship between activity and helical structure, further detailed investigation is required.

The facts discussed above can be summarized by using Vallee's notation (*9*), with some modifications, as follows:

$$[(\mathrm{TAA})\mathrm{Ca}]\mathrm{Ca}_n \underset{\text{crystallization}}{\overset{\text{dissolution}}{\rightleftarrows}} n\mathrm{Ca}^{++}+[(\mathrm{TAA})\mathrm{Ca}], \quad (n=9 \text{ or } 10)$$

and

$$[(\mathrm{TAA})\mathrm{Ca}] \underset{\text{neutralization}}{\overset{\text{acidification}}{\rightleftarrows}} \mathrm{Ca}^{++}+(\mathrm{TAA}) \longrightarrow (\mathrm{TAA})'.$$

Here, 〔(TAA)Ca〕 represents the full active and stable TAA molecule*, (TAA) the inactive and reversibly denatured molecule, and (TAA)′ an irreversibly denatured protein.

The fact that the inactivation of TAA by EDTA always proceeds irreversibly (*1*, *10*) may also be accounted for as follows. The addition of EDTA to 〔(TAA)Ca〕 causes the elimination of the essential calcium from the enzyme protein just as in the case of acid inactivation. The elimination reaction, however, proceeds at a considerable slow rate, and under such a circumstance the irreversible formation of (TAA)′ predominates over the calcium elimination reaction. It renders the reactivation of (TAA) almost impossible. This relation may be more easily understandable by the following expression:

$$[(\mathrm{TAA})\mathrm{Ca}] \overset{\substack{\text{EDTA}\\ \text{(slow)}}}{\rightleftarrows} \mathrm{Ca}^{++} + (\mathrm{TAA}) \xrightarrow[\text{(fast)}]{} (\mathrm{TAA})'.$$

Besides the maintenance of active configuration of protein moiety of TAA, it remains to be answered whether or not the calcium has another role, *i.e.*, responsibility for the formation of enzyme-substrate complex.

## SUMMARY

1. The firmly bound calcium atom in TAA is able to exchange in solution with free exogenous calcium ions but not with strontium ions.

2. During repeated crystallization procedure the calcium atom exchanges not only with calcium but also with strontium and magnesium ions. The cxchange rate is greatest with calcium and smallest with magnesium.

3. The preparations of TAA produced by *A. oryzae* grown in the media containing no added calcium was found to possess a considerable amount of non-dialysable calcium which might have been derived from impurities.

4. The TAA protein has a strong affinity to calcium, although the bound calcium can be replaced by strontium without loss of the enzymatic activity.

5. During the acid inactivation of TAA and its reactivation by neutralization, the enzymatic activity and the amount of non-exchangeable calcium show a good parallelism.

6. A similar relationship was observed between the enzymatic activity and the change in levo-rotatory power or dispersion constant which may be regarded as a measure of denaturation.

7. The possible role of the essential calcium atom in the maintenance of enzymatic activity of TAA was discussed.

The author wishes to express his gratitude to Prof. S. Akabori for his kind guidance, and to Sankyo Co., Ltd. for their kind supply of "Takadiastase Sankyo". The author

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* TAA is protected from heat inactivation by various salts, especially, calcium salt. In this respect, 〔(TAA)Ca〕 is less stable in the absence of free calcium ion. The stabilility mentioned here is true in low or at most, ordinary temperature.

also wishes to thank Otozai Laboratory, Facultfy of Science, Osaka University, for the determination of metals.

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# THE STRUCTURE OF SOME PEPTIDES IN TAKA-AMYLASE A

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(Received for publication, September 5, 1958)

The chemical nature of Taka-amylase A (TAA) has been intensively studied by Akabori and his coworkers (*1–7*). The determinations of amino acid composition (*8*) and amino- (*9*) and carboxylterminal amino acids (*10*) of crystallized TAA were already reported.

The present paper deals with the structure of some peptides containing lysine or aromatic amino acids which were obtained by partial hydrolysis of dinitrophenylated Taka-amylase A (DNP-TAA).

TAA was dinitrophenylated as usual, and the resulting DNP-TAA was partially hydrolysed by hydrochloric acid and the hydrolysate was fractionated by the adsorption chromatography using talk-celite column which selectively adsorbed ε-DNP-lysine peptides (Fr. 1). The effluent was chromatographed on activated carbon, which adsorbed peptides containing aromatic amino acids (Fr. 2).

These fractions were treated by the countercurrent distribution (CCD) method, and the peptides corresponding to the peaks in the distribution patterns were further purified by column chromatography. Among these peptides, three could be characterized as valyl-(lysine, glutamic acid)-aspartic acid, phenylalanyl-aspartic acid and leucyl-aspartyl-tyrosine.

## EXPERIMENTALS

*Materials*—About 5 g. of crystalline Taka-amylase A, which was prepared from "Takadiastase Sankyo" and recrystallized three times from water-acetone system (*1*, *2*), was dissolved in 50 ml. of water followed by addition of 4 g. of sodium bicarbonate and 3 ml. of 2,4-dinitrofluorobenzene in 100 ml. of ethanol. The mixture was shaken for 3 hours at room temperature, and then acidified. The precipitate formed was centrifuged, washed successively with water, alcohol and ether, and finally dried *in vacuo* (*9*). DNP-TAA obtained was about 5.1 g.

*Partial Hydrolysis of DNP-TAA*—DNP-TAA (4.0 g.) was dissolved in 50 ml. of 12 *N* hydrochloric acid, and kept standing for 20 hours at 40°. Under these conditions, DNP-TAA was hydrolysed up to about 50 per cent of the total hydrolysis as shown in Fig. 1.

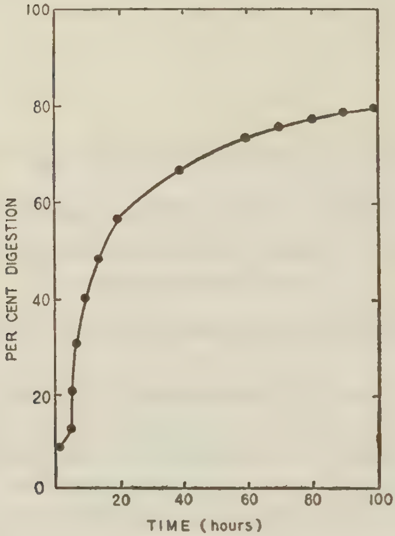

**Fig. 1.** Libration of amino-terminal in Taka-amylase A in the course of digestion with 12 *N* HCl.

*Adsorption of the Peptides Containing ε-DNP-lysine*—The partially hydrolysed DNP-TAA was diluted with 550 ml. of water and extracted with ethyl acetate. The ethyl acetate layer seemed to involve DNP-peptides which was formed from the amino end in TAA. One third of the aqueous layer was applied to the column chromatography with the talk-celite column, which was 8 cm. in diameter and consisted of 500 g. talk and 200 g. of celite (Hyflo Super-cell) (*11*). The peptides containing ε-DNP-lysine were absorbed forming yellow bands on the column. After the column was washed with 800 ml. of 1 *N* HCl, the effluent and the washing were brought together, evaporated *in vacuo* and kept for further treatment. The adsorbed DNP-peptides were eluted out with the solvent composed of 1 *N* HCl and ethanol (1 : 4 in volume ratio). The effluent was evaporated to dryness *in vacuo* below 40° and 1.55 g. of DNP-peptides (Fr. 1) were obtained.

*Adsorption of the Peptides That Contained Aromatic Amino Acids*—The mixture of peptides which passed through the talk-celite column was dissolved in 300 ml. of water and mixed with the activated carbon*. (*12*) After filtration, it was washed twice with 300 ml. of water. The carbon which adsorbed the peptides was suspended into the solution composed of phenol, acetic acid and water (1 : 4 : 15 in volume), shaken for 2 hours filtered again and washed twice with 500 ml. of the same solvent. This solution was extracted with ether in order to remove phenol, and then the aqueous layer was evaporated *in vacuo* below 40°. About 2 g. of the residue was obtained (Fr. 2).

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* About 5 g. of carbon suspended in 600 ml. of 5 per cent acetic acid aqueous solution (*v/v*) was shaken for 2 hours, filtered, and washed three times with 600 ml. of water.

Absorption curves of these two fractions both in 1 per cent HCl are shown in Fig. 2.

*Further Fractionation by CCD Method*—In order to fractionate further, Fr. 1 and Fr. 2 were treated by CCD (13)* method.

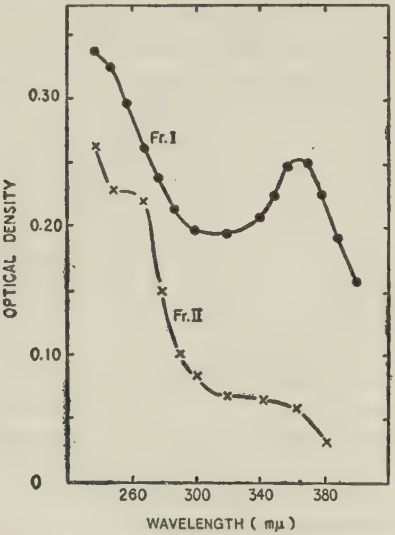
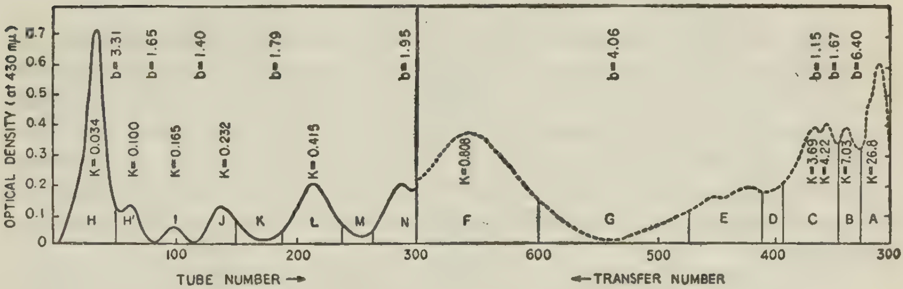


FIG. 2. Absorption curves of the fractionated peptides from the partial hydrolysate of Taka-amylase A.



Solvent system Iso-propylether : *n*-butanol : 0.125 *N* HCl (1 : 1 : 2)
Procedures Foundamental procedure : 300
Single withdrawal procedure : 400
All procedure : 700

FIG. 3. Countercurrent distribution curve of partial hydrolysate (Fr. 1).

Fr. 1 was distributed over 700 stages (300 fundamental procedures and 400 single withdrawal procedures), with the solvent system ; iso-propyl ether : *n*-butanol : 1/8 *N* HCl (2 : 1 : 1 *v*/*v*/*v*) and the distribution curve obtained is shown in Fig. 3.

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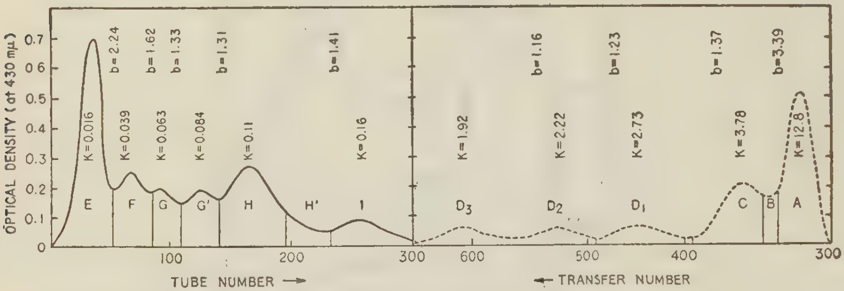
* The full automatic counter-current distribution apparatus was employed, and this apparatus, which contains 300 tubes, was made in our laboratory according to Craig's report (*14*).

The sample (0.6 g.) was taken into the first 4 tubes. After distribution the quantity of DNP-peptide in each tubes was determined by spectrophotometry at 430 m$\mu$.

Fr. 2 was dinitrophenylated by the usual method (*15*) and distributed over 1500 transfers which involved 300 fundamental procedures, 300 single withdrawal procedures and 3 times recycling procedure (900 steps) using the solvent system composed of benzene, chloroform, methanol and 1/100 *N* HCl (0.5 : 2 : 2 : 3 *v*/*v*/*v*/*v*). The distribution curve is also shown in Fig. 4.



Solvent system Chloroform : benzene : methanol : 0.01 *N* HCl (2 : 0.5 : 2 : 3)
Procedures Foundamental procedure : 300
Single withdrawal procedure : 350
Recycling procedure : 900
All procedure : 1550

**Fig. 4.** Countercurrent distribution curve of partial hydrolysate (Fr. 2).

The distribution patterns obtained from spectrophotometric analysis of the tubes at 430 m$\mu$ are shown in Figs. 3 and 4, and the solution corresponding to each peak in these curves separately evaporated under mild condition.

*Determination of the Structure of Fr. 1-J*—The Fr. 1-J in the first distribution pattern (Fig. 3) was further purified by celite or silica gel column chromatography. The result suggested that the fraction was still composed of three components. The main band Fr. 1-$J_1$ was used for the following experiment.

The peptide of this band was hydrolysed by 6 *N* HCl for 7 hours at 100°. The hydrolysate was extracted with ether in an acidic medium. The ether layer was colorless and the aqueous layer was yellow. The latter was treated by silica gel and celite column chromatography and the yellow substance was identified as ε-DNP-lysine. This aqueous layer was also chromatographed by paper, with *n*-butanol ; acetic acid ; water (4 : 1 : 1) and 80 per cent phenol aqueous solution. After spraying ninhydrin reagent, four purple spots were obtained and identified as valine, aspartic acid, glutamic acid and ε-DNP-lysine. The last one was faintly yellow before the treatment with ninhydrin.

The starting material was dinitrophenylated by the usual method, purified by column chromatography, and hydrolysed by 6 *N* HCl at 100° for 12 hours.

The DNP-amino acid extracted with ether from the acidic solution was identified as DNP-valine by silica gel column chromatography. The presence of ε-DNP-lysine was also confirmed by paper chromatography. In another experiment amino acids in this aqueous phase were dinitrophenylated and identified by column chromatography as DNP-glutamic acid, DNP-aspartic acid and di-DNP-lysine. Each of these bands on silica gel column was eluted and determined spectro-photometrically at 430 mμ. These results are summarized in Table I.

For determination of the carboxyl terminal of this peptide, the dinitrophenylated peptide was hydrazinolysed by anhydrous hydrazine at 100° for 5 hours, and the hydrazinolysate was dinitrophenylated as already reported in the previous papers (*16*, *17*). By these treatments, two bands were observed on the silica gel column, one of which was determined as DNP-aspartic acid and the other seemed to be di-DNP-derivative of acidic amino acid monohydrazide. The latter was hydrolysed by 6 *N* HCl at 100° for 3 hours, the resulting hydrolysate was identified as DNP-glutamic acid, and the original DNP-derivative is supposed to be di-DNP-glutamic acid monohydrazide. From all of these experiments, which are schematically shown in Scheme 1, the peptide, Fr. 1-$J_1$ is suggested to be valyl-(ε-DNP-lysine, glutamic acid)-aspartic acid, but the order of the peptide sequence lysine, glutamic acid was not yet determined.

TABLE I

*Amino Acid Composition of Purified Peptides*

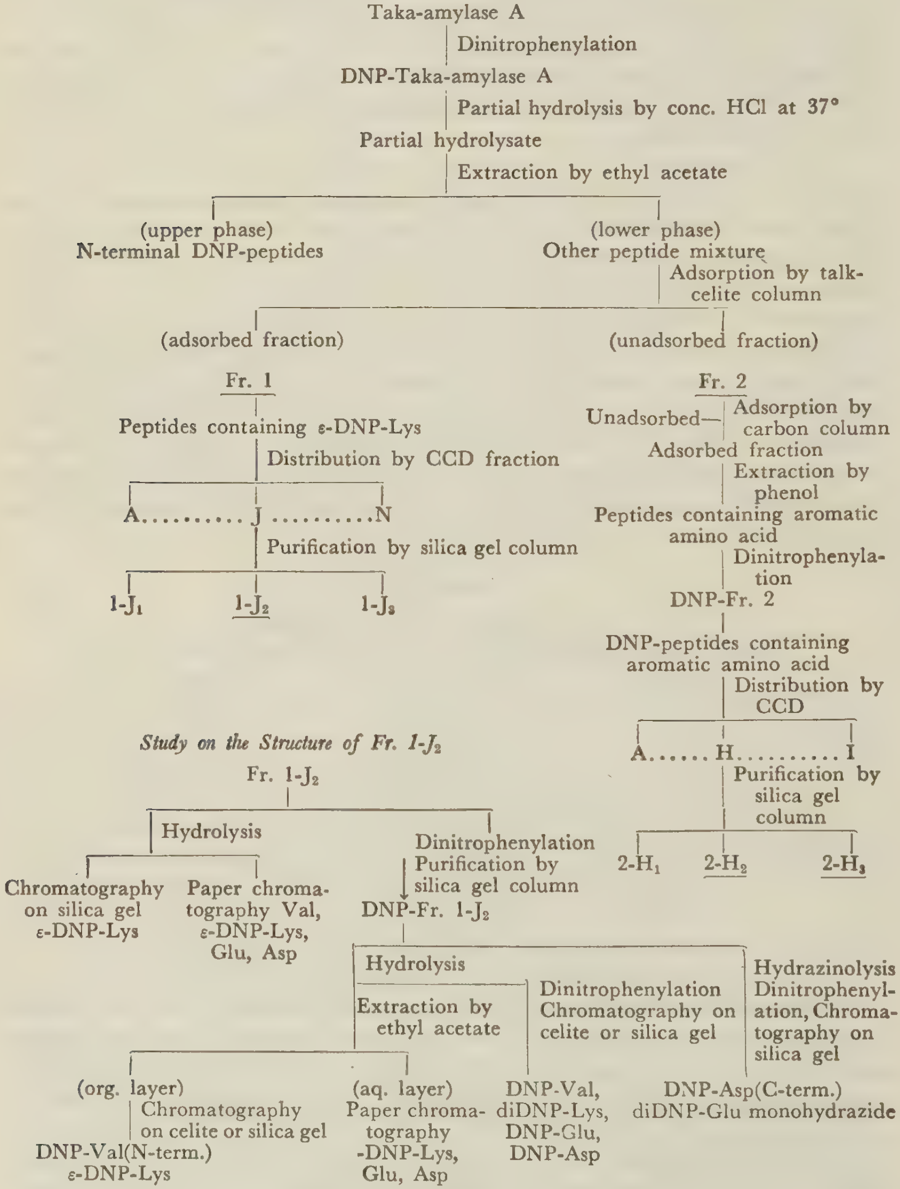
| Peptide No. | DNP-amino acid | Mole ratio |
|---|---|---|
| Fr. 1-J | DNP-Val<br>diDNP-Lys<br>DNP-Glu<br>DNP-Asp | 1.00<br>0.91<br>1.09<br>0.82 |
| Fr. 2-$H_2$ | DNP-Phe<br>DNP-Asp | 1.00<br>0.81 |
| Fr. 2-$H_3$ | DNP-Leu<br>diDNP-Tyr<br>DNP-Asp | 1.00<br>0.77<br>0.89 |

*Determination of the Structnre of Fr. 2-H*—The counter-current distribution curve of Fr. 2 is shown in Fig. 4. Fr. 2-H was not pure and could be fractionated into three components by column chromatography. Of these components, two main fractions Fr. 2-$H_2$ and Fr. 2-$H_3$, were used for characterization of the peptides.

Fr. 2-$H_2$ was hydrolysed 6 *N* hydrochloric acid at 100° for 20 hours, and DNP-amino acid was extracted from the hydrolysate by ether. DNP-phenylalanine was characterized as amino terminal amino acid by silica gel and celite chromatography. The aqueous layer was examined by paper chromatography and was determined to be aspartic acid. After the hydrolysate

## SCHEME 1

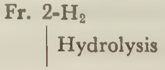
*Schematic Purification and Determination Procedure of Partial Hydrolysate of TAA*

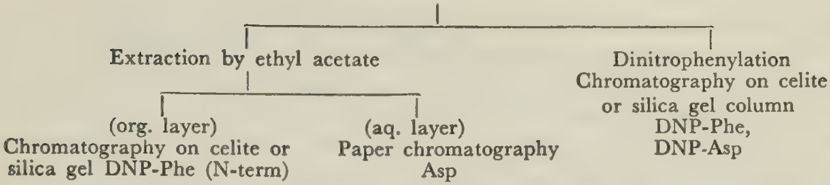


*Study on the Structure of Fr. 1-J₂*

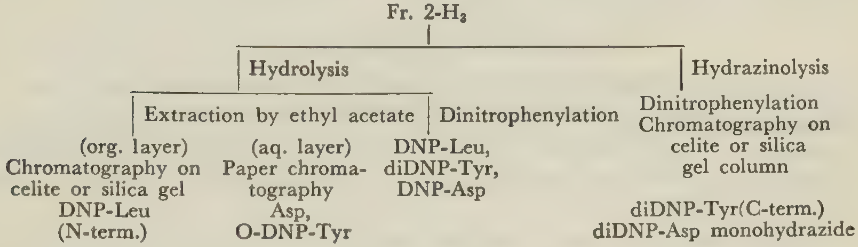
Taka-amylase A
Dinitrophenylation
DNP-Taka-amylase A
Partial hydrolysis by conc. HCl at 37°
Partial hydrolysate
Extraction by ethyl acetate
(upper phase)
N-terminal DNP-peptides
(lower phase)
Other peptide mixture
Adsorption by talk-celite column
(adsorbed fraction)
(unadsorbed fraction)
Fr. 1
Peptides containing ε-DNP-Lys
Distribution by CCD fraction
A.......... J ..........N
Purification by silica gel column
1-J₁
1-J₂
1-J₃
Fr. 2
Unadsorbed—
Adsorption by carbon column
Adsorbed fraction
Extraction by phenol
Peptides containing aromatic amino acid
Dinitrophenylation
DNP-Fr. 2
DNP-peptides containing aromatic amino acid
Distribution by CCD
A...... H.......... I
Purification by silica gel column
2-H₁
2-H₂
2-H₃
Study on the Structure of Fr. 1-J₂
Fr. 1-J₂
Hydrolysis
Chromatography on silica gel ε-DNP-Lys
Paper chromatography Val, ε-DNP-Lys, Glu, Asp
Dinitrophenylation
Purification by silica gel column
DNP-Fr. 1-J₂
Hydrolysis
Extraction by ethyl acetate
(org. layer)
Chromatography on celite or silica gel
DNP-Val(N-term.)
ε-DNP-Lys
(aq. layer)
Paper chromatography
-DNP-Lys, Glu, Asp
Dinitrophenylation
Chromatography on celite or silica gel
DNP-Val, diDNP-Lys, DNP-Glu, DNP-Asp
Hydrazinolysis
Dinitrophenylation, Chromatography on silica gel
DNP-Asp(C-term.)
diDNP-Glu monohydrazide

*Study on the Structure of Fr. 2-H₂*



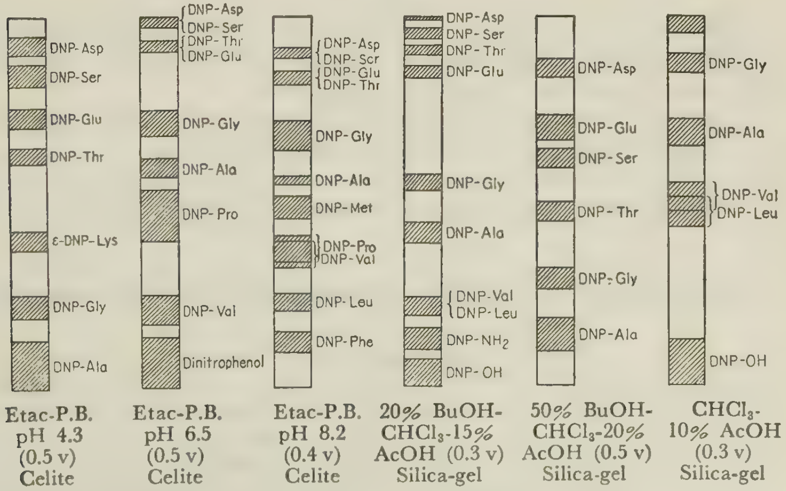

Extraction by ethyl acetate
(org. layer)
Chromatography on celite or silica gel DNP-Phe (N-term)
(aq. layer)
Paper chromatography
Asp
Dinitrophenylation
Chromatography on celite or silica gel column
DNP-Phe,
DNP-Asp

### *Study on the Structure of Fr. 2-H$_3$*



was dinitrophenylated and chromatography, DNP-phenylalanine and DNP-aspartic acid were detected to be in the ratio 1 to 1 as shown in Table I. From the results of these experiments, it may be concluded that Fr. 2-H$_2$ is DNP-phenylalanyl-aspartic acid.

The another main component, Fr. 2-H$_3$, was also characterized by the same procedure, and DNP-leucine was shown to be amino-terminal amino acid, *O*-DNP-tyrosine and aspartic acid belonging to the other component amino acids. Dinitrophenylation of the hydrolysate of Fr. 2-H$_3$ gave DNP-



P.B. = Phosphate buffer (1 M) BuOH = *n*-Butanol
Etac = Ethyl acetate AcOH = Acetic acid

FIG. 5. Column chromatography of DNP-amino acid.

leucine, di-DNP-tyrosine and DNP-aspartic acid in the ratio shown in Table I. By the hydrazinolysis of this peptide, followed by the dinitrophenylation di-DNP-tyrosine, was determined as carboxyl terminal amino acid. From these studies, which are also shown schematically in Scheme 1, the structure of Fr. 2-$H_3$ may be concluded to be DNP-leucyl-aspartyl-*O*-DNP-tyrosine. Some instances of silica gel or celite (545) column chromatogram of amino acid are shown in Fig. 5.

## RESULTS AND DISCUSSION

The countercurrent distribution method seems to be very useful for the separation of DNP-peptides especially in a large quantity. For the complete separation of the peptides it was necessary to use other purification methods such as column chromatography in addition to the countercurrent distribution method.

Combining the above described methods three peptides have been separated in fairly pure state and their amino acid compositions and sequences were studied. These are valyl-(lysine, glutamic acid)-aspartic acid, phenylalanyl-aspartic acid, and leucyl-aspartyl-tyrosine. In the determination of carboxyl-terminal amino acid of these peptides, the hydrazinolysis method has been found most convenient. The complete sequence of the tetrapeptide has not yet been established, but the fact that glutamyl-aspartic acid sequence was frequently found in natural proteins suggests the probable structure to be valyl-lysyl-glutamyl-aspartic acid.

## SUMMARY

1. The partial hydrolysates of DNP-Taka-amylase A were investigated.

2. Two fractions were obtained by using the adsorption chromatography with talk-celite column and with activated carbon.

Each fraction was further fractionated by countercurrent distribution method combined with celite or silica gel chromatography.

3. The structure of three peptides were elucidated to be valyl-(lysine, glutamic acid)-aspartic acid, phenylalanyl-aspartic acid and leucyl-aspartyl-tyrosine.

The authors are grateful to Sankyo Co., Ltd. for supplying them ‘ Takadiastase Sankyo ’.

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# ACTIONS OF RADIATIONS ON ENZYMES

## I. BIOCHEMICAL EFFECTS OF GAMMA RADIATION ON UREASE*

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(Received for publication, September 15, 1958)

The action of various types of radiation on living cells, is said to be attributed to the biochemical effects caused by ionizing radiations on certain constituents or systems of biological importance, especially such as enzymes and enzyme systems. It is expected that by irradiation with X-rays, $\gamma$-rays, $\alpha$-particles, $\beta$-particles, protons and neutrons, the chemical changes which take place in certain atomic groups in the enzyme molecules are such as to cause inhibition of their activity. Dale's pioneer work on the inactivation of carboxypeptidase (*1*, *2*), and several other studies on urease (*3*, *4*) have already been carried out as to the effects of X-rays and other radiations. In the studies on inactivation of enzyme by X-rays, Barron and his collaborators (*4*, *5*) showed that enzymes which have active sulfhydryl groups were susceptible to inhibition by moderate doses of X-rays and this inhibition was attributable to the oxidation of the sulfhydryl groups to inactive disulfides.

In the present work, the mechanisms causing the inactivation of urease, one of the well known sulfhydryl enzymes, by $\gamma$-ray irradiation were studied.

### EXPERIMENTALS

*Preparation of Urease*—Urease was prepared from the jack bean meal of reddish species harvested in Japan**, which was ground by a stone mortar in order to avoid inhibitory damage to the activity of the enzyme according to the methods described essentially by Sumner (*8*), by Hellerman (*9*) and by Dounce (*10*). With 1 liter of 31.5 per cent aldehyde-free acetone, 200 g. of jack bean meal, of which the ureolytic activity had been found to be 220 units per g., were extracted at 22° for 10 minutes with gentle stirring. The mixture was filtered by filter paper (Toyo Roshi, No. 5-C) on a Buchner funnel. To 760 ml. of the filtrate were added 190 ml. of pure acetone. After the mixture was allowed to stand overnight at 0°, it was centrifuged at about 3,000 r.p.m. for 20 minutes. The precipitates obtained were suspended in 10 ml. of redistilled water. The suspension was again centrifuged at about 3,000 r.p.m. for 20 minutes. The precipitates were extracted with 20 ml. of redistilled water under occasional stirring and allowed to stand for some hours, and then centrifuged. By repetition of the extraction, 75 ml. of the supernatant liquid, which were found to contain 15,600 units of the ureolytic activity per g. of protein, were obtained. To this crude urease solution enough amounts of pure acetone were added to make a 31 per cent acetone solution. This mixture was left to stand overnight at 0°, and centrifuged at about 3,000 r.p.m. for 30 minutes. The sediment was extracted with 20 ml. of redistilled water, and reprecipitated with acetone. The same treatment was repeated three times. Finally crystals of urease* were obtained and collected by centrifuging at 10,000 r.p.m. for 30 minutes at 0°. It was found that the crystals have urease activity of 68,000 units per g. of protein.

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* Parts of this article were reported at the 30th Annual Meeting of the Biochemical Society of Japan in Kyoto, 1957 (*6*), and at the 2nd Symposium on Atomic Energy of Japan in Tokyo, 1958 (*7*).

** Octahedral crystalls of urease, which have been usually obtained from the American jack bean meal were reported to be hard to isolate from the Japanese specimens by several investigators. (Nagai, Y., and Yamazaki, M., personal communications.)

*Estimation of Urease Activity*—Ureolytic activity was determined by the procedure described by Van Slyke (*11*) with some modifications. Original colorimetric measurements of pH were replaced by direct pH determinations using a Beckman GS pH meter. In this paper activities of urease were expressed by the Sumner unit: 200 Sumner units corresponding to the activity causing a pH-shift from 6.7 to 7.7 in 5 minutes at 20° when 0.5 ml. of 10 per cent enzyme solution was mixed with 5 ml. of 5 per cent urea solution in 0.2 $M$ phosphate buffer.

*Irradiation of $\gamma$-Rays*—$\gamma$-Rays used for irradiation were: 50 c. of $Co^{60}$ in Toshiba RIT-I** Teletherapy Unit and 200 c. in Toshiba Irradiation Apparatus*** equipped for technical uses.

Urease preparations were held in a glass tube of 0.8 cm. in diameter and 5 cm. in length or in sealed tubes of 1 ml. to 20 ml. in volume, and were irradiated at 25 to 27° in air. The dose rate of $\gamma$-irradiation was 50 kr. per hour.

*Measurement of Radiation Doses*—Chemical dosimetry using ferrous sulphate (*12*, *13*) was employed to determine the radiation dose. Solutions of $10^{-3}$ $M$ ferrous ammonium sulphate in 0.8 $N$ $H_2SO_4$ were irradiated by $\gamma$-rays, and the optical absorption of the ferric sulphate produced in the solutions was estimated at 301 m$\mu$ by a Beckman DU spectrophotometer. For calculating the dose rate, an ionic yield of 15.5 mole ferric ion per 100 e.v. was used.

## RESULTS AND DISCUSSION

Air dried jack bean meal, its suspension in water, and crude and purified urease preparations from the bean meal were irrradiated with doses of $\gamma$-rays from 17 kr. to 800 kr. respectively. The inactivation occurring in the ureolytic activity of urease in the preparations is shown in Table I.

The results show that the enzyme is more radio-sensitive in a state with much water than in a dry state and also that the more advanced the purification of the enzyme, the more it becomes sensitive against strongly it was affected by $\gamma$-rays. Participations of the irradiation products of water are strongly assumed in this type of inhibition as supposed by other workers (*14*, *15*).

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* Although the crystals of this enzyme preparation appeared uniformly shaped under microscope, two separate peaks were found in its electrophoretic pattern (unpublished data).

** Equipment in the University Hospital of Kyoto was used; The authors express their gratitude to Prof. T. Fukuda, Kyoto University, for his permission to use it.

*** Equipment in the Chemical Research Institute of Kyoto University was used; The authors wish to express their thanks to Prof. I. Sakurada and Prof. S. Okamura, Kyoto University, for their permission to use this equipment.

At a certain concentration of urease solution, the inhibition of urease activity was observed to take place exponentially with the increase of doses of γ-rays, as shown in Table II.

### TABLE I

*Inactivation of Urease in Various States by γ-Rays*

| Enzyme preparation | Dose (*kr.*) | Enzyme activity | | Inhibition (%) |
|---|---|---|---|---|
| | | Control (*unit*) | Irradiated (*unit*) | |
| Jack bean meal[1)] | 17<br>800 | 170<br>170 | 170<br>140 | 0<br>18 |
| Jack bean meal suspension[2)] | 17<br>800 | 200<br>200 | 200<br>100 | 0<br>50 |
| Crude urease solution[3)] | 17<br>800 | 430<br>430 | 360<br>0 | 16<br>100 |
| Pure urease solution[4)] | 17<br>800 | 480<br>480 | 0<br>0 | 100<br>100 |

Enzyme preparation contained the following amount of urease preparations respectively, 1) 50 mg. in a dry state, 2) 100 mg. per ml. in redistilled water, 3) 20 mg. per ml. in redistilled water, 4) 10 mg. per ml. in redistilled water.

Each in a small glass tube was irradiated with γ-rays of $Co^{60}$ at 800 r. per minutes of dose. Enzyme activity is given in Sumner units.

### TABLE II

*Effect of γ-Rays on Urease. Relation between Dose and Extent of Inactivation of Urease*

| Enzyme preparation | Dose (*kr.*) | Enzyme activity | | Inhibition (%) |
|---|---|---|---|---|
| | | Control (*unit*) | Irradiated (*unit*) | |
| Pure urease solution[1)] | 0.8<br>2.5<br>8.3<br>17.0 | 480<br>480<br>480<br>480 | 480<br>370<br>260<br>0 | 0<br>22.9<br>45.8<br>100 |
| Crude urease solution[2)] | 8.3<br>16.6<br>25.0<br>50.0<br>100.0<br>200.0 | 280<br>280<br>280<br>280<br>280<br>280 | 280<br>190<br>180<br>120<br>50<br>24 | 0<br>32.2<br>35.7<br>57.2<br>82.2<br>91.5 |

Enzyme solution contained the following amount of urease preparations respectively, 1) 10 mg. per ml., 2) 11.2 mg. per ml., in redistilled water.

Irradiation procedure was identical with that in Table I.

On plotting the percentage of inhibition, therefore, against the logarithm of dose, a straight line is obtained (Fig. 1).

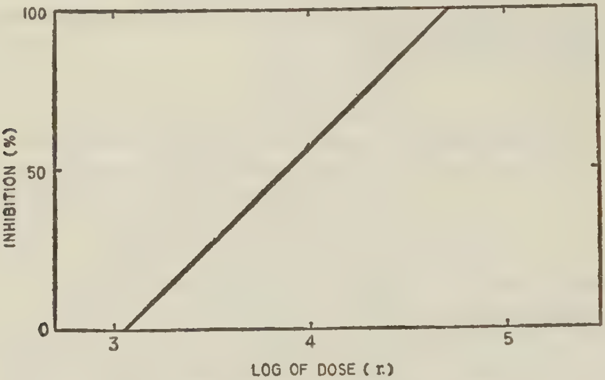

FIG. 1. Inactivation of urease by $\gamma$-rays.

Enzyme solution contained 8 mg. of crystalline urease per ml. of redistilled water. Irradiation was carried out by the same procedure as in Table I.

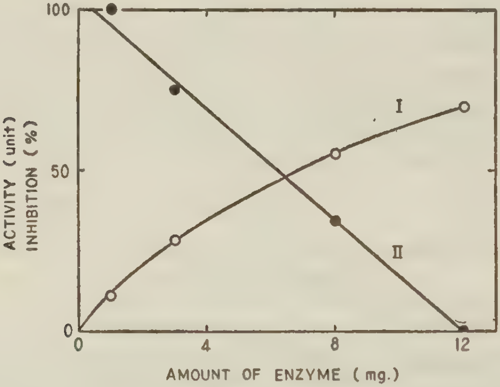

FIG. 2. Effect of $\gamma$-rays on urease. Relation between enzyme activity and concentration of enzyme solution.

The measured amount of crystalline urease was always dissolved in 0.5 ml. of a buffer solution. The dose of $\gamma$-irradiation was 8 kr. for 10 minutes. Curve I and II show the relationships between activity and concentration and also between inactivation and concentration, respectively.

This inactivation of urease in aqueous solution, however, was not always proportional to the sort of concentration of the enzyme solutions. As shown in Fig. 2, in a dilute solution it is more sensitive to $\gamma$-rays than in a concentrated solution.

The fact that in the purer states and also in the more dilute solutions urease is more sensitive to $\gamma$-irradiations suggests that the enzyme may be protected competitively by some impurities coexisting or by the inactivated enzyme proteins produced by $\gamma$-irradiations, as proposed by Dale (*15*).

The protective effects of several substances such as sodium chloride, glycine, ovalbumin and reduced glutathione are shown in Table III, and that of reduced glutathione are given in Table IV.

TABLE III

*Protective Effect of Various Substances on Urease against $\gamma$-Irradiation*

| Compound | Concentration (*M*) | Activity of urease after treated (*unit*) | Inactivation of urease (%) | Protection of enzyme inactivation (%) |
|---|---|---|---|---|
| None | — | 263 | 26.3 | — |
| Sodium chloride | 0.85 | 270 | 24.4 | 7.5 |
| Glycine | 0.60 | 286 | 19.9 | 24.5 |
| Ovalbumin | $1.1\times10^{-3}$ | 294 | 17.7 | 33.0 |
| Glutathione (reduced) | $4.0\times10^{-3}$ | 328 | 8.3 | 69.2 |

To 0.1 ml. of aqueous solution containing 4 mg. of crystalline urease, was added 0.4 ml. of the testing solution. The solution in a glass tube was irradiated with the dose of 50 kr. of $\gamma$-rays during one hour. Activity of urease after irradiation was compared with 357 units of non-irradiated enzyme activity.

TABLE IV

*Protection of Urease Activity from $\gamma$-Inhibition by Reduced Glutathione (GSH)*

| Dose (*kr.*) | Without GSH | | Treated with GSH | | Protection (%) |
|---|---|---|---|---|---|
| | Activity (*unit*) | Inhibition (%) | Activity (*unit*) | Inhibition (%) | |
| 0 | 280 | — | 240 | — | — |
| 17 | 200 | 28.6 | 260 | 0 | 100.0 |
| 50 | 100 | 64.3 | 220 | 16.7 | 74.0 |

Urease solution contained 5.6 mg. of enzyme preparation in 0.5 ml. of redistilled water and reduced glutathione in final concentration of 0.001 *M*.

The activity of enzyme solutions irradiated is compared with that of solutions containing no glutathione.

Although the mechanism of protecting action exerted by the above-mentioned compounds except glutathione is not yet clear, the action of reduced glutathione is intelligible. The reactive thiol groups of glutathione probably compete with sulfhdryl groups in the enzyme molecule against the effective action of the irradiated products of water.

The reactivation experiments of partially inactivated ureased by thiol compounds such as reduced glutathione and cysteine are presented in Table V, and the results are also attributable to the same cause proposed by Barron *et al.* (*4*, *5*, *16*).

TABLE V

*Reactivation of γ-Irradiated Urease with Glutathione (GSH) and Cysteine (CSH)*

| Enzyme solution | Dose (*kr.*) | Activity after irradiation | | Inhibition | | Reactivation (%) |
|---|---|---|---|---|---|---|
| | | Without GSH or CSH (*unit*) | With GSH or CSH (*unit*) | Before addition of GSH or CSH (%) | After addition of GSH or CSH (%) | |
| I | 8.3 | 280 | 310 | 0 | 0 | 100 |
| | 16.6 | 190 | 230 | 32.2 | 17.9 | 45 |
| | 25.0 | 180 | 220 | 35·7 | 21.4 | 40 |
| | 50.0 | 120 | 120 | 57.2 | 57.2 | 0 |
| II | 0.8 | 480 | 480 | 0 | 0 | 100.0 |
| | 2.5 | 370 | 430 | 23.0 | 10.4 | 54.8 |
| | 8.3 | 260 | 290 | 45.9 | 39.6 | 13.8 |

As the enzyme solution (I) 8.6 mg. of a crude urease preparation in 0.5 ml. of buffer solution which had 280 units of activity and reactivated with glutathione were used. For (II), 5 mg. of crystalline urease in 0.5 ml. of buffer solution which had 480 units and reactivated with cysteine were used. The final concentration of reduced glutathione in enzyme solution was 0.001 *M* and that of cysteine was 0.0005 *M*. Immediately after irradiation under the same condition as shown in Table I, the thiol compound was added to the enzyme solution, and the activity was compared with that of the enzyme solution irradiated in the absence of the thiol compounds.

By γ-irradiation of water, hydrogen atoms, hydrogen ions, hydrated electrons and hydroxyl radicals are proved to be formed as the primary product (*17*, *18*).

In the presence of dissolved oxygen the hydrogen atoms combine with oxygen molecules to form $O_2H$ radicals. Hydrogen peroxide are also produced from hydroxyl radicals by their mutual recombination process. Among these primary and secondary irradiation products of water, hydroxyl and $O_2H$ radicals and also hydrogen peroxide are considered to have a strong oxidizing power. Sulfhydryl groups in the enzyme molecule (ESH) have been well known to be very sensitive to oxidizing agents and easily changeable into corresponding disulfides (ES-SE) by dehydrogenation.

Thus, oxidation reactions of sulfhydryl groups by the irradiated products of water may be pictured as follows (*19*):

$$2ESH + 2OH \longrightarrow ES\text{-}SE + 2H_2O$$
$$2ESH + 2O_2H \longrightarrow ES\text{-}SE + 2H_2O_2$$
$$2ESH + H_2O_2 \longrightarrow ES\text{-}SE + 2H_2O$$

The disulfide groups thus formed may be again reduced to the original forms by reduced glutathione (GSH) or cysteine (CSH):

$$\mathrm{ES{-}SE} + 2\mathrm{GSH} \rightleftarrows 2\mathrm{ESH} + \mathrm{GS{-}SG}$$
$$\mathrm{ES{-}SE} + 2\mathrm{CSH} \rightleftarrows 2\mathrm{ESH} + \mathrm{CS{-}SC}$$

This is the mechanism of inactivation of the sulfhydryl enzyme in an aqueous solution by irradiation and also of its reactivation by thiol compounds.

Among various factors affecting the inactivation of enzyme by radiations, one of the most important conditions may be the pH of the enzyme solution, which is closely related to enzyme activity. In the range of pH from 4.3 to 9.1, effects on urease caused by $\gamma$-rays were tested and the results were presented in Table VI. It was found that urease was most stable at its optimum pH so far as examined.

### Table VI

*Effect of pH on the Inactivation of Urease by $\gamma$-Rays*

| Buffer | Control | | Irradiated | | Inhibition (%) | pH of treated solution* |
|---|---|---|---|---|---|---|
| | pH | Activity (*unit*) | pH | Activity (*unit*) | | |
| 0.1 *N* Acetate | 4.3 | 0 | 4.3 | 0 | — | 7.0 |
| „ | 5.0 | 330 | 5.0 | 160 | 51.5 | 7.2 |
| 0.1 *N* Phosphate | 6.6 | 360 | 6.8 | 300 | 16.7 | 7.4 |
| » | 7.5 | 380 | 7.5 | 340 | 10.6 | 7.4 |
| » | 7.8 | 180 | 7.9 | 160 | 11.2 | 7.2 |
| 0.2 *N* Borate | 9.1 | 110 | 9.1 | 60 | 65.5 | 6.9 |

Enzyme solution containing 6 mg. crystalline urease in 0.5 ml. of a buffer solution was used. The dose of $\gamma$-irradiation was 8 kr. for 10 minutes.

* For determination of the activity, pH of the enzyme solution was buffered by 0.2 *M* phosphate buffer, pH 7.0, containing 3 per cent urea.

### Table VII

*Ammonia Produced from Urease Solutions by $\gamma$-Irradiations*

| Dose (*kr.*) | Ammonia (*$\mu$g./ml.*) | Ammonia-N formed per total-N of urease (%) |
|---|---|---|
| 3 | 0 | — |
| 30 | 0.7 | 0.01 |
| 50 | 2.5 | 0.05 |
| 100 | 4.8 | 0.10 |
| 200 | 10.5 | 0.21 |
| 400 | 12.5 | 0.25 |
| 800 | 19.3 | 0.38 |

Enzyme solution contained 26.2 mg. of crystalline urease per ml. of distilled water. The dose rate was 50 kr. per hour. The amount of ammonia was determined by the method described by Conway (*21*) and Kock *et al.* (*22*).

In its dilute solution, inactivation of urease by relatively larger doses of $\gamma$-rays was found to be more difficult to be reversed (Table V). This type of inactivation may be due to the chemical change or changes in the enzyme molecule other than oxidation of the sulfhydryl groups. When aqueous solutions of urease were irradiated by large doses of $\gamma$-rays, production of appreciable amounts of ammonia was observed as shown in Table VII.

Oxidative deamination of several amino acids caused by $\gamma$-irradiation and the radiolytic breakdown of peptides are already proposed by the authors (*20*). The similar breakdown of enzyme molecules and the following deaminative process are probably causative of the formation of ammonia by $\gamma$-irradiation. The principal caused of irreversible inactivation of urease by larger doses of $\gamma$-radiation is concluded to be decompositions of component amino acids of urease molecules.

## SUMMARY

1. The inactivating mechanism of enzyme by X-ray irradiations proposed by Dale and Barron *et al.* was reconfirmed with the effect of $\gamma$-rays on urease.

2. Urease is proved to be more sensitive in its aqueous solution than in an air dried state.

3. Urease in aqueous solutions is found to be protected against $\gamma$-ray inactivation by the presence of several substances such as sodium chloride, glycine, ovalbumin, especially reduced glutathione and cysteine.

4. By small doses of $\gamma$-irradiations inactivation of urease is reversibly relieved by the reducing agents, such as reduced glutathione or cysteine. In this case, oxidation of essential sulfhydryl groups in the enzyme molecules by radiation products of water is causative of its inactivation.

5. Urease is less sensitive against $\gamma$-ray at pH at which the enzyme shows its optimal activity.

6. Larger doses of $\gamma$-ray irradiations cause formation of ammonia in urease solutions and consequent irreversible inactivation of the enzyme activity. Inactivation of this type is attributed to chemical changes occurring in the enzyme molecule, such as the radiolytic breakdown of peptide bonds followed by the oxidative deamination of its component amino acids.

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# ROTATORY DISPERSION OF NATIVE AND DENATURED TAKA-AMYLASE A

By AKIO MAEDA* AND ATSUSHI OIKAWA**

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(Received for publication, September 15, 1958)

In the previous paper (*1*), it was reported that Take-amylase A (TAA) has ten atoms of calcium per molecule, and one of them is essential for maintaining the active configuration of TAA. This conclusion was based on the fact that the elimination of the last calcium atom from the TAA molecule by the treatment with ethylenediamine tetraacetic acid (EDTA) caused an increase of levo-rotatory power of this protein. The optical rotatory power, however, is supposed to be affected by environmental conditions in addition to the intrinsic helical configuration of protein molecule. Thus, it is interesting to examine the rotatory dispersion which is an excellent measure for estimating the degree of folding of protein molecules. The present paper deals with the results obtained from the experiments on rotatory dispersion of TAA.

## MATERIALS AND METHODS

*Native TAA*—Three times recrystallized TAA (*2*) was dissolved in distilled water.

*Dialysed TAA*—Native TAA was dialysed in a cellophane tubing against 0.02 *M* sodium acetate in a cold room. The dialysate was renewed every day and the dialysis continued for 4 days to eliminate non-essential calcium atoms (*1*).

*EDTA-treated-TAA*—Native TAA was dialysed in a cellophane tubing against 0.02 *M* sodium acetate containing 0.0025 *M* EDTA (pH 7) in a cold room. The dialysate was renewed every day. The preparation finally obtained after dialysis in the presence of EDTA was found to contain 0.3 equivalent of calcium, and its enzymatic activity was 28 per cent of its original value.

*Urea-denatured TAA*—A TAA solution was mixed with 4 volumes of 10 *M* urea and the mixture was incubated at 30° for 13 days. The levo-rotation of the solution increased and reached eventually a constant value. The preparation finally obtained had no enzymatic activity, indicating that this preparation was completely denatured.

The rotatory power of TAA was measured by using a Rudolph polarimeter Model 80 which consisted of a Beckman spectrophotometer and a Rudolph photoelectric polarimeter attachment. Measurements were made at the wave-lengths, 365.0, 404.1, 435.8, 491.6,

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546.1, 577.0 and 589.3 m$\mu$; these were obtained from a mercury lamp except the last one which was the D line of sodium lamp.

Although all of the TAA solutions were not buffered, their pH values remained at about 7. While the temperature was not controlled, it was within the range from 12° to 15° throughout the measurements. The polarimeter tube used was 20 cm. in length. Other methods were entirely the same as in the previous report (*1*) except that the enzymatic activity was measured at 30°.

## RESULTS

At first, the rotatory dispersion of TAA was measured with the solutions of three different concentrations, *i.e.*, 48, 24 and 12 mg. TAA per ml. (Fig. 1). Hewitt observed (*3*) that Drude's one-term equation, $[\alpha]_\lambda = k_c/(\lambda^2-\lambda_c^2)$, is applicable to for the rotatory dispersion of the protein. In this equation, $\lambda$ stands for the wave-length, $[\alpha]_\lambda$ for the specific rotation at the wave-length $\lambda$, and $\lambda_c$ and $k_c$ are constants. Rewriting it as $1/[\alpha]_\lambda = (\lambda^2-\lambda_c^2)/k_c$, $1/[\alpha]_\lambda$ is proportional to $(\lambda^2-\lambda_c^2)$. In the present experiments,

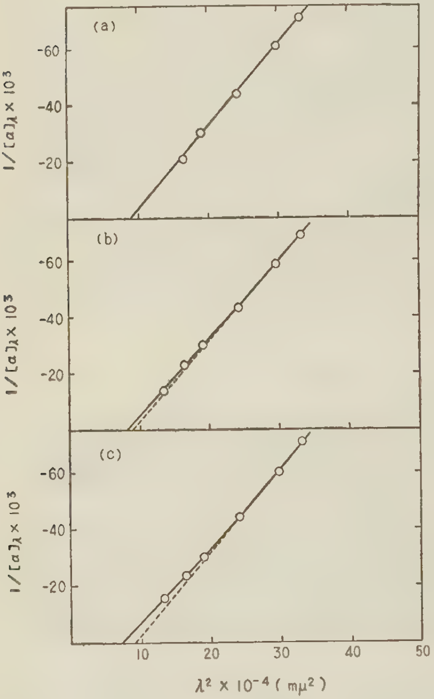

FIG. 1. Rotatory dispersion of various concentrations of native TAA. (A) 48, (B) 24, (C) 12 mg. TAA per ml.

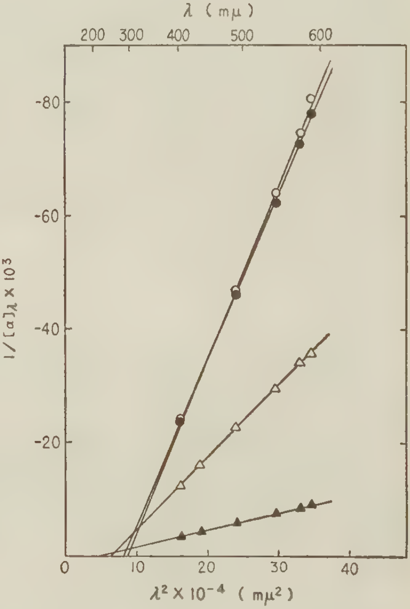

FIG. 2. Rotatory dispersion of native, dialysed, EDTA-treated and urea-denatured TAA. —○—, native; —●—, dialysed; —△—, EDTA-treated; —▲—, urea-denatured TAA.

the constants, $\lambda_c$ and $k_c$, were calculated from the experimental data by the least square method to minimize the deviation of each plot from the theoretical curve. When measurement was made on the solution, the concentration of which was 48 mg. TAA per ml., its rotatory dispersion seemed to obey Drude's one-term equation, and $\lambda_c$ was calculated to be 298 m$\mu$. But deviations from this equation were observed with the solutions of lower protein concentrations. From the values for longer wave-lengths, $\lambda_c$ was calculated to be 300 m$\mu$, while the values for shorter wave-lengths a value of 270 m$\mu$ was obtained for $\lambda_c$. Therfore, the measurements hereafter were carried out with the solutions containing 40 to 50 mg. of TAA per ml.

The rotatory dispersion of the TAA preparations treated under various conditions is shown in Fig. 2, and their $\lambda_c$ values and other proterties are recorded in Table I.

TABLE I

*$\lambda_c$ and other Properties of Native, Dialysed, EDTA-treated and Urea-denatured TAA Solutions.*

| TAA | pH | $[\alpha]_D$ | $\lambda_c$ (*m$\mu$*) | Ca content (*g. atom per mole TAA*) | Enzymatic Activity (*S.P. per $\mu$g TAA* at 30°) |
|---|---|---|---|---|---|
| Native | 7.15 | −12.4 | 293 | 10.6 | 3.08 |
| Dialysed | 7.10 | −12.8 | 290 | 1.3 | 3.10 |
| EDTA-treated | 7.10 | −28.1 | 257 | 0.3 | 1.45 |
| Urea-denatured* | — | −113 | 221 | — | 0 |

* Optical rotatory power was measured in the urea-containing solution.

Native and dialysed TAA showed almost the same $\lambda_c$ value. The $\lambda_c$ value of the EDTA-treated TAA was considerably lower than that of native one, though higher than that of urea-denatured TAA.

## DISCUSSION

The values of $\lambda_c$ and $[\alpha]_D$ have so far been measured by several authors with a number of proteins (*4*, *5*). These data indicate that most of the native proteins show $\lambda_c$ values from 245 to 270 m$\mu$ and $[\alpha]_D$ from −30 to −70°. It is also concluded by these authors that the denaturation of a protein is generally accompanied by an increase of its levo-rotation and a shift of its $\lambda_c$ value to shorter wave-lengths. TAA is, however, unique in these respects; its $\lambda_c$ value is as high as 290 m$\mu$ and when treated with EDTA it is reduced to a value usually obserbed for native proteins.

Recently Doty and his colleagues suggested (*5*, *6*) that a high $\lambda_c$ value is a measure of the high helix content of a protein molecule. It is, therfore, reasonable to assume that TAA has an unusually high helix content in aqueous solution, and this value reaches the level exhibited by some proteins only in non-aqueous solutions (*5*).

These facts can be explained by assuming that the TAA molecule freed from the essential calcium has a folded structure comparable to that of usual proteins and that the insertion of calcium caused a super-organization of the molecule to a more complex and active structure.

## SUMMARY

1. Rotatory dispersion of native, dialysed, EDTA-treated and urea-denatured TAA was measured.

2. Native TAA has an unusually high $\lambda_c$ value as compared with other proteins.

3. The role of calcium was discussed based on the rotatory behavior of TAA.

The authors wish to express their thanks to Prof. S. Akabori for his interest and encouragement throughout this work, and to Sankyo Co., Ltd. for kind supply of Takadiastase Sankyo.

*Addendum in proof.* Dr. B. Jirgensons has recently published a paper (*Arch. Biochem. Biophys.* **74,** 70 (1958)) in which the $\lambda_c$ value of take-amylase A was determined to be 261.3 m$\mu$ at pH 6.84. The amylase preparation used was supplied to him from this laboratory in a lyophilized state. The low $\lambda_c$ value obtained by him may have been due to partial denaturation of the amylase protein caused by lyophilization.

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# STUDIES ON THE CHEMISTRY OF WAX D OF BCG

## I. FRACTIONATION OF WAX D OF BCG: ISOLATION OF CORD FACTOR AND OLIGOMANNOINOSITIDES

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"Purified wax", as isolated by Anderson (*1*), was a complex lipide prepared from acid fast bacteria by extraction with chloroform. Some of its hydrolytic products were chemically identified by him and his collaborators.

Recently, Asselineau (*2*) fractionated "purified wax" into Wax C and Wax D. Wax C was a mixture of high molecular compounds of low melting points, which contained mycolic acids and carbohydrates but no nitrogeneous compounds as constituents, while Wax D was reported to consist mainly of high molecular lipopolysaccharides of higher melting points containing small amounts of phosphorus and nitrogen.

Following the first report that the toxic factor of tubercle bacilli known as "cord factor" was concentrated in Wax C fraction (*3*), chemical investigations of the constituents in that fraction were performed intensively, chiefly by the use of column chromatography, with the results that cord factor and some other complex lipides were isolated in pure forms and their characteristics were clarified (*4*).

Wax D, on the other hand, has not been investigated as fully as Wax C (*5*). Wax D, as well as similar preparations, seems to play a biologically significant role in manifesting toxicity of tubercle bacilli (*6*), in causing tuberculin allergy as an adjuvant (*7*), and in other related phenomena (*8*). However, there are many conflicting views (*9*) on these biological activities. Such contradictory views indicate the need for better methods for resolution of complex lipides in the fractions and chemical characterization of the individual component.

It was previously shown by the present author that Wax D of BCG usually contained 3 percent of inositol (*10*). The present paper describes a method for fractionating Wax D of BCG into two components by silicic acid column chromatography. One fraction was a lipide mixture of low melting point, lacking phosphorus and nitrogen, from which "cord factor" (trehalose-6,6'-dimycolate) was isolated. Another fraction consisted of a mixture of lipides of relatively high melting point containing phosphorus and inositol. From the latter fraction were isolated new phosphoinositides containing manno-oligo-ose as a constituent. These phosphoinositides have been tentatively designated as "*Oligomannoinositides*."

## MATERIALS AND METHODS

*Sugar* was estimated according to the anthrone reaction using mannose as standard (*11*). If it was necessary to discriminate between hexose and pentose, *total sugar* was estimated by trypthophan method (*12*) and *hexose* was determined by cysteine sulphuric acid method (*12*). *Glycerol* was determined by Blix's (*13*).

*Paperchromatography of sugar* was performed at room temperature using Whatman No. 1 and either of the following solvent mixtures, (i) 80 per cent aqueous phenol, (ii) *n*-butanol-pyridine-water (6 : 4 : 3) or (iii) ethyl acetate-acetic acid-water (3 : 1 : 3). The spots were detected by spraying with aniline oxalate solution and ammoniacal silver nitrate solution.

Other materials and methods have been described in a preceding paper (*10*).

## RESULTS

### *Column Chromatography of Wax D of BCG with Silicic Acid*

As shown in Table I, chromatographic separation of Wax D from *BCG* furnished two fractions, the characteristics of which were quite different from each other. "A fraction," eluted with chloroform only and with chloroform containig 5 per cent methanol, gave two mixtures of compounds of low melting points containing little phosphorus, nitrogen or inositol. On the other hand, "B fraction," eluted with chloroform containing more than 10 per cent methanol and with methanol only, consisted of compounds which melted higher than 170°, which contained phosphorus and inositol.

TABLE I

*Chromatography of 1.0 g. of Wax D of BCG Dissolved in Chloroform, Absorbed on 20 g. of Silicic Acid and 10 g. of Hyflosupercel*

| Fraction No. | Tube No. | Eluted with 200 ml. of | Weight (*mg.*) | *m.p.* (°C) | P (%) | N (%) | Glycerol (%) | Inositol (%) |
|---|---|---|---|---|---|---|---|---|
| Original | | | 1000 | 140–170 | 0.80 | 0.20 | 3.62 | 3.18 |
| A | 1–2 | Chloroform | 230 | 38 – 40 | 0.0 | 0.06 | 3.32 | 0.00 |
| | 3–4 | Chloroform-methanol 95 : 5 | 190 | 42 – 43 | 0.1 | 0.11 | 2.34 | 0.00 |
| B | 5–6 | Chloroform-methanol 90 : 10 | 230 | 178 | 1.52 | 0.11 | 5.20 | 7.11 |
| | 7 | Chloroform-methanol 80 : 20 | 60 | 170–172 | 1.76 | 0.14 | 5.40 | 6.46 |
| | 8 | Methanol | 100 | >220 | 1.48 | 0.20 | | 4.87 |

Wax D from other lots of *BCG* also furnished similar elution patterns by silicic acid column chromatography and the yields of Fraction A and B were fairly constant in all preparations (Table II). The fact that the total recovery of Wax D after chromatographic partitioning showed unvariably 80 to 90 per cent may indicate the firm adsorption of the lipides to the adsorbent.

TABLE II

*Yields of Fraction A and Fraction B Separated by Chromatography in Wax D of Different Lots of BCG*

| Lot No. | Wax D (*g.*) | Silicic acid (*g.*) | Yield of A (%) | Yield of B (%) | A+B |
|---|---|---|---|---|---|
| 1 | 1.0 | 20 | 42 | 39 | 81 |
| 2 | 3.9 | 50 | 33 | 53 | 86 |
| 3 | 5.6 | 80 | 51 | 39 | 90 |
| 4 | 1.3 | 30 | 38 | 55 | 93 |

### *Chromatography of Fraction A by Mg-silicate and Silica Gel: Identification of Cord Factor*

The fact that Fraction A was similar to Wax C with respect to melting point and lack of nitogeneous components and inositol suggested the former could be further fractionated by the partition chromatographic procedure of Noll and Bloch (*14*). Using this procedure, Noll, Bloch, Lederer

TABLE III

*Chromatography of 1.349 g. of Fraction A of Wax D (BCG) Dissolved in Petroleum Ether, Absorbed on 20 g. of Magnesium Silicate (Florisil) and 10 g. of Hyflosupercel*

| Fraction No. | Eluted with 200 ml. of | | *mg.* | m.p. (°C) |
|---|---|---|---|---|
| 1—2 | Petroleum ether | | 12 | 60—70 |
| 3—5 | Petroleum ether-benzene | 9:1 | 79 | 47—54 |
| 6—7 | Petroleum ether-benzene | 9:1 | 18 | |
| 8—9 | Petroleum ether-benzene | 1:1 | 73 | 43—46 |
| 10—11 | Petroleum ether-benzene | 1:1 | 14 | |
| 12—13 | Benzene | | 33 | 43—46 |
| 14 | Benzene | | 2 | |
| 15 | Benzene-ether | 9:1 | 40 | 44—45 |
| 16 | Benzene-ether | 9:1 | 4 | |
| 17—19 | Benzene-ether | 1:1 | 22 | 42—45 |
| 20—21 | Ether | | 13 | |
| 22—25 | Ether-methanol | 9:1 | 818 | 43 |
| 26 | Ether-methanol | 9:1 | 32 | |
| 27—28 | Ether-methanol | 8:2 | 62 | 43—46 |
| | Recovered | | 1222 (91% of A) | |

and Asselineau (*4*) were able to isolate cord factor and other complex lipides from Wax C. This chromatographic technique was applied to Fraction A in the same manner, giving the result shown in Table III. About 67 per cent of Fraction A was concentrated in the ether-methanol eluate. After romoval of the solvent from this fraction, the residue was dissolved in a small amount of chloroform, then precipitated with methanol to obtain 670 mg. of a white powder melting at 43∼46°.

A sample of 670 mg. of this powder was dissolved in 50 ml. of petroleum ether, chromatographed on a column of 40 g. of silica gel and further eluted with 200 ml. of petroleum ether. As a result, 570 mg. of a waxy substance was obtained from the total petroleum ether fraction. After resolution of the substance in petroleum ether and re-precipitation with methanol, a white powder was obtained melting at 43° (cord factor from *BCG* has a m.p. at 43∼45°). The yield was 26 per cent from Wax D.

| | C | H | |
|---|---|---|---|
| Calcd. for $C_{186}H_{366}O_{17}$: | 77.70 | 12.83 | per cent |
| Found: | 77.80 | 12.46 | 〃 |
| | 77.70 | 12.90 | 〃 |

The infrared spectrum of this compound showed a series of well defined bands all characteristic to cord factor (Fig. 1). The exact positions of the absorption maxima expressed in cm.$^{-1}$ are shown below in comparison with those of cord factor shown by Noll and Bloch (*14*):

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Data from Noll: | 3350 | 2900 | 2840 | 1715 | 1470 | 1380 | 1275 | 1170 | |
| Present data : | 3360 | 2915 | 2845 | 1710 | 1465 | 1368 | 1280 | 1165 | |
| Data from Noll: | 1150 | 1100 | 1075 | 1050 | 1020 | 995 | 915 | 805 | 720 |
| Present data : | 1140 | 1098 | 1065 | 1048 | 1013 | 985 | 925 | 790 | 711 |

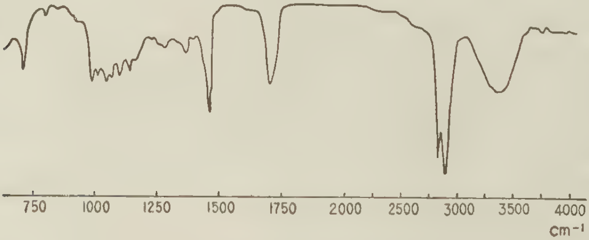

Fig. 1. Infrared spectrum of cord factor isolated from Wax D of *BCG* (taken from melt).

### *Alkaline Hydrolysis of the Compound Melting at 43°*

The compound, 186 mg., was suspended in a soluiton of 200 mg. of KOH in 5 ml. of methanol and 5 ml. of benzene. The solution was refluxed for 1.5 hours. After cooling, the solvent was removed at room temperature and to the resulting residue was added gradually a small amount of ice-cold water. The aqueous solution was neutralized with 1 *N* HCl and repeatedly extracted with ether. The combined ether extracts were evaporated to dryness and 163 mg. of the lipide residue was dissolved in 10 ml. of *n*-butanol and 10 ml. of 1.5 *N* alcoholic KOH. The solution was refluxed for 4 hours. After cooling, 50 ml. of water was added and butanol was removed under reduced pressure. The resulting aqueous solution was extracted several times with ether and the combined ether extracts were taken to dryness. The residue dissolved in benzene was then chromatographed on a column of 5 g. of alumina (Brockmann), washed with benzene and ether and finally eluted from the column with ether containing 10 per cent of glacial acetic acid. From the eluate, 121 mg. of residue was obtained, melting at 53∼55°. This substance, 9.915 mg., dissolved in *n*-butanol was titrated with 0.735 ml. of 1/100 *N* of methanolic NaOH (F=1.049) using BTB as an indicator.

| | mol. wt. |
|---|---|
| Calcd. for mycolic acid ($C_{87}H_{174}O_4$) (*2*): | 1284 |
| Found: | 1285 |

The water-soluble portion obtained after extraction of mycolic acids was freed from salts by passing through Dowex-2 ($CO_3^{--}$) and IR-120 ($H^+$) and concentrating *in vacuo* to a syrup. This was dissolved in 6 ml. of 1 *N* HCl, heated for 2 hours in a boiling water bath, cooled, again passed through IRA-400 ($CO_3^{--}$) and concentrated *in vacuo* to a small volume. Paper chromatography of this solution using 80 per cent aqueous phenol gave only one spot with an $R_f$ value 0.36 which was identical with that of glucose. From the experiments above described, it can be concluded that the substance of m.p. 43° is cord factor (trehalose-6, 6'-dimycolate) (*15*).

### *Comparison of Various Preparations of Fraction B from Wax D of Different Lots of BCG*

Table IV shows a comparison of some analytical data of Fraction B from Wax D of different lots of *BCG*. Sugars in this fraction, as detected by paper chromatography, were mannose and a very small amount of arabinose. From this fraction, new phosphoinositides containing various amounts of mannose as their components were isolated as one of the main constituents. The composition and the structure of "*Oligomannoinositides*" will be reported in the next paper (*18*).

## DISCUSSION

Silicic acid chromatographic partitioning with chloroform and methanol as eluting solvents has been successfully applied to the phosphatides of various origins in nature (*16*). Wax D of *BCG* was also fractionated by this same technique into two components. The fact that cord factor was isolated from one fraction of Wax D leads the author to the following opinion.

TABLE IV

*Comparison of Some Analytical Data of Fraction B from Wax D of Different Lots of BCG*

| Lot | m.p. (°C) | P (%) | Glycerol (%) | Inositol (%) | Mannose (%) | Fatty acids (%) | Mannose[4] | Arabinose[4] |
|---|---|---|---|---|---|---|---|---|
| 1-a | 178[1] | 1.52 | 5.20 | 7.11 | 20.6 | | ⧻ | + |
| -b | 172[2] | 1.76 | 5.40 | 6.46 | | | ⧻ | + |
| -c | 220[3] | 1.48 | | 4.87 | 20.6 | | ⧻ | trace |
| 2 | 193[2] | 1.90 | | 9.68 | 36.6 | 40 | ⧻ | trace |
| 3-a | 190[3] | 1.51 | 4.70 | 6.40 | 46.7 | 39.0 | ⧻ | trace |
| -b | 205[3] | 1.40 | | | 51.0 | 42.2 | ⧻ | trace |
| -c | 214[3] | 1.20 | | | 37.9 | | ⧻ | trace |
| 4 | 205[3] | 0.83 | | | 87.9 | | ⧻ | trace |

1) Eluted with chloroform containing 10 per cent of methanol.
2) Eluted with chloroform containing 20 per cent of methanol.
3) Eluted with chloroform containing 50 per cent of methanol.
4) Detected by paper chromatography with aniline oxalate as spray reagent.

Reports previously published all pointed out the existence of cord factor in Wax C of *BCG* as well as of human type tubercle bacilli and the actual isolation of cord factor was reported (*15*). In this experiments, however, cord factor was prepared from Wax D in a yield of approximately 0.36 per cent as calculated on the dry weight basis of *BCG*. The result is quite in accordance with the date presented by Noll (*14*). Because of the small yield of Wax C and also of cord factor when "purified wax" was extracted with boiling acetone, he dissolved "purified wax" in benzene and precipitated Wax D with acetone leaving Wax C in the supernatant. This extraction technique raised the yield of Wax C to 60~90 per cent of "purified wax". On the other hand, Tsumita (*17*) was unable to isolate cord factor from Wax C of *BCG* prepared by boiling acetone procedure. The present experiments together with the results shown by Noll and by Tsumita strongly indicate that cord factor is mainly concentrated in Wax D instead of Wax C when "purified wax" is extracted with boiling acetone.*

As for Fraction B, it is mainly composed of glyceroinositophosphatides containing mannose from the analytical data of Table IV. But the amounts of mannose in Fraction B varies from one lot to another. The molecular ratios between mannose and phosphorus in Lot 1-c, Lot 3-b and Lot 4 are 2.4, 5.7 and 18.3, respectively. In this respect, it is of great interest that in Fraction B there appeared to be phosphoinositides containing some manno-oligo-ose different from the mannobiose, which is a constituent of phosphatidylinositodimannoside, the structure of which was proposed by Lederer. A proof to support the chemical combination between manno-oligo-ose and phosphoinositides will be given in the next paper (*18*).

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* In this report, Wax C and Wax D were originally defined as such that Wax C is soluble in hot acetone and Wax D insoluble in boiling acetone (*19*).

In the present experiments, it was impossible to isolate so-called "*Lipopolysaccharides*" which were reported to be composed mainly of mycolic acids and polysaccharides (*5*). Although the present study did not examine all the fractions of Wax D of *BCG* two possibilities may be suggested: (1) "Lipopolysaccharides" may be found only in "purified wax" from the human type strain and not in that from *BCG* or (2) they may be present in fractions which were not investigated; specifically, those eluted from silicic acid with chloroform containig 10 to 20 per cent methanol or in the fraction firmly adsorbed on the column amounting to about 10 per cent of Wax D. Which of these possibilities is right remains to be ascertained.

Finally, the fact that Wax C is a mixture of various neutral lipides such as cord factor, glycerol monomycolate, phthiocerol dimycolate and so on (*4*, *2*, *17*), suggests it should be possible to fractionate "purified wax" directly by silicic acid column chromatography.

## SUMMARY

Wax D of *BCG* was fractionated into two constituents by silicic acid chromatography. From one fraction, cord factor was isolated and from the other, phosphoinositides containing oligomanno-ose, tentatively designated as "*Oligomannoinositides*," were obtained. The behaviour of "purified wax" on the silicic acid column was discussed.

The author wishes to thank Dr. D. Mizuno, Chief of the Department of Chemistry, National Institute of Health, Tokyo, for his constant encouragement and advices throughout this work. He is also grateful to Professor Dr. T. Ukita, of the Faculty of Pharmaceutical Sciences, University of Tokyo, for his constant encouragement. Thanks are also due to Miss E. Kondo, of the Department of Chemistry, National Institute of Health, for her skillful technical assistance in every experiment of this work. Microanalyses for carbon and hydrogen were carried out by Miss S. Hara of the Department of Chemistry, National Institute of Health, Tokyo.

*Addenda:* Since this manuscript was submitted it has come to the attention of the author that lipopolysaccharides have been isolated from Wax D of human type strain of tubercle bacilli. They were found in the chloroform eluate from the partition chromatography of Wax D on silicic acid. (Personal communication from Dr. Asselineau (Paris))

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# THE SYNTHESIS OF STERO-BILE ACIDS

## XXXII. THE PARTIAL SYNTHESIS OF TRIHYDROXY-24-METHYLCOPROSTANIC ACID

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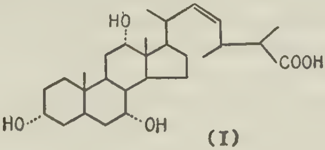
(Received for publication, October 9, 1958)

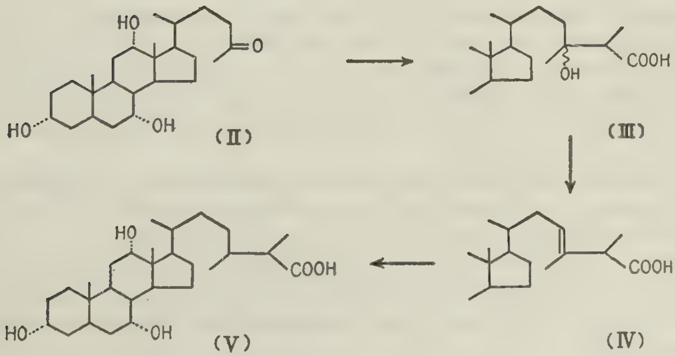
As a result of the comparative studies on the bile salt component in the bile of various vertebrates by Shimizu and co-workers, several kinds of stero-bile acids and bile-sterols were isolated from the bile of the animals of low class such as frog and toad.

Formation of the stero-bile acids and bile-sterols in various vertebrates is of a considerable biogenetic interest, since these compounds might represent intermediate forms from the sterols to the normal bile acids.

Shimizu, Oda and Kazuno (*1*, *2*) isolated trihydroxybufosterocholenic acid (m.p. 160°) and trihydroxyisosterocholenic acid (m.p. 227°), having a probable formula of $C_{28}H_{46}O_5$, from the bile of the toad (*Bufo vulgaris formosus*). Shimizu and Kazuno (*3*, *4*) classified later the nuclear structure and the location of the double bond of these acids, from the fact that both the isomeric acids were converted to bisnorcholic acid



(I)



by ozonization. Although the structure of the terminal part of their side chain has not yet been disclosed, the following structure (I) has been proposed tentatively by the authors.

Various stero-bile acids have been synthesized in our laboratory in order to elucidate the chemical structure of the natural stero-bile acids (*5*), but the structure of trihydroxybufosterocholenic acid and trihydroxyisosterocholenic acid remained still unknown.

The author, therefore, attempted to synthesize 3α, 7α, 12α-trihydroxy-24-methylcoprostanic acid which has the same structure as Shimizu and Kazuno (*3*, *4*) suggested previously.

## EXPERIMENTAL

*3α, 7α, 12α, 24-Tetrahydroxy-24-methylcoprostanic Acid* (III)—Six g. of norcholylmethylketone (II) and 25 g. of ethyl DL-α-bromopropionate were added to a mixture of 30 ml. of dry toluene and 10 ml. of dry benzene with 40 g. of granulated zinc and a few crystals of iodine. When the mixture was refluxed on a sand bath, a vigorous reaction set in and the solution became cloudy. Gently refluxing was continued for 1.5 hours under rapid stirring, and then the reaction mixture was cooled. The condensation product was decomposed with 200 ml. of 10 per cent sulfuric acid and the separated benzene-toluene layer was evaporated and extracted with ether. The ether extract was washed with sodium thiosulfate solution and water, dried and then the solvent was evaporated under a reduced pressure. The brownish oily residue was hydrolyzed with 150 ml. of 5 per cent methanolic potassium hydroxide for 2 hours on a water bath. After evaporation of methanol from the hydrolysate under addition of certain ml. of water, the alkaline solution was extracted with ether repeatedly in order to remove the unchanged methylketone, and then acidified with dilute hydrochloric acid to obtain a precipitate. The precipitate was extracted with ethyl acetate. The extract was washed with water, dried, and the solvent was evaporated to dryness. The oily residue was esterified with ethereal diazomethane solution and purified by chromatography on a alumina column. Removal of the solvent from the ethereal elute gave 5 g. of crystalline methyl 3α, 7α, 12α, 24-tetrahydroxy-24-methylcoprostanate, m.p. 144°.

*3α, 7α, 12α-Trihydroxy-24-methylcoprostenic Acid* (IV)—Five g. of the crude methyl 3α, 7α, 12α, 24-tetrahydroxy-24-methylcoprostanate were dissolved in 50 ml. of 80 per cent formic acid and heated for 5 hours at 55–60°.

The reaction mixture was poured into water and a yellowish precipitate was obtained. After the precipitate was digested with ether, the ether layer was washed with water, dried, and the solvent was evaporated to dryness. The oily residue was added to 50 ml. of freshly distilled thionyl chloride. After the mixture was let stand for 2 hours, excess thionyl chloride was distilled off under a reduced pressure, and the brownish oily residue was treated with petroleum ether.

This oily chloride was dissolved in 30 ml. of dimethylaniline and refluxed on a sand bath for 20 minutes. After cooled, the reaction mixture was dissolved in ether, and the ether solution was washed with water, dilute hydrochloric acid and water successively, and then dried. Evaporation of the solvent left 4.2 g. of viscous oily residue, which were dissolved in 200 ml. of 15 per cent methanolic potassium hydroxide. After hydrolysis for 2 hours on a water bath, the hydrolysate was poured into water, and concentrated to drive methanol off.

The alkaline solution was filtered so as to remove insoluble neutral substance. The filtrate was acidified with dilute hydrochloric acid to obtain a precipitate, which was digested with ethyl acetate. The ethyl acetate solution was washed with water, dried and concentrated under a reduced pressure.

Crystallization of the residue from ethyl acetate gave 0.5 g. of crude 3α, 7α, 12α-trihydroxy-24-methylcoprostenic acid (IV). Several recrystallizations from ethyl acetate, gave a pure sample, m.p. 182° (130° sinter). Yield 420 mg. of prisms.

*Analysis* Calcd. for $C_{28}H_{46}O_5\frac{1}{2}H_2O$: C 71.30, H 10.05
Found: C 71.59, H 10.03

*3α, 7α, 12α-Trihydroxy-24-methylcoprostanic Acid* (V)—Three hundred and eighty mg. of the above 3α, 7α, 12α-trihydroxy-24-methylcoprostenic acid (IV) dissolved in glacial acetic acid were hydrogenated with 230 mg. of platinum oxide catalyst at room temperature. Theoretical volume of hydrogen was absorbed within 5 minutes. After removal of the platinum catalyst from the reaction mixture, the solvent was evaporated to dryness.

Recrystallization of the residue from ethyl acetate gave 300 mg. of rods, m.p. 186–188°. After several recrystallizations from ethyl acetate, a pure sample of 3α, 7α, 12α-trihydroxy-24-methylcoprostanic acid (V) m.p. 192°, was obtained, giving a positive Hammarsten reaction.

*Analysis* Calcd. for $C_{28}H_{48}O_5$: C 72.37, H 10.41
Found: C 72.21, H 10.16

*Methyl 3α, 7α, 12α-Trihydroxy-24-methylcoprostanate*—A solution of 3α, 7α, 12α-trihydroxy-24-methylcoprostanic acid (V) in methanol was treated with ethereal diazomethane solution. After 2 hours, excess diazomethane and the solvent mixture were driven off by gentle heating.

Recrystallizations from ethyl acetate yielded methyl 3α, 7α, 12α-trihydroxy-24-methylcoprostanate, needles, m.p. 172°.

*Analysis* Calcd. for $C_{29}H_{50}O_5$: C 72.76, H 10.53
Found: C 72.32, H 10.19

*Methyl 3, 7, 12*-Triketo-*24*-methylcoprostanate—To a solution of 150 mg. of methyl 3α, 7α, 12α-trihydroxy-24-methylcoprostanate in 3 ml. of glacial acetic acid, a solution of 150 mg. of chromic anhydride in 0.3 ml. of water and 3 ml. of glacial acetic acid was added. After being allowed to stand at room temperature for an hour, the mixture was diluted with water, and the resulting precipitate was extracted with ethyl acetate. The ethyl acetate solution was washed with water, dried, and the solvent was distilled off.

Recrystallizations from ethanol gave needles, m.p. 218–220° of methyl 3, 7, 12-triketo-24-methylcoprostanate.

*Trioxime of Methyl 3, 7, 12-Triketo-24-methylcoprostanate*—To a solution of 50 mg. of methyl 3, 7, 12-triketo-24-methylcoprostanate in absolute ethanol, was added a hydroxylamine acetate solution prepared from hydroxylamine hydrochloride and potassium acetate.

The mixture was refluxed for 5 hours, diluted with water, white crystals being precipitated.

Recrystallizations from methanol yielded needles, m.p. 230°, of trioxime of methyl 3, 7, 12-triketo-24-methylcoprostanate.

## RESULTS AND DISCUSSION

Condensation of norcholylmethylketone (II) (*6*) with ethyl DL-α-bromopropionate by modified Reformatsky reaction gave 3α, 7α, 12α, 24-tetrahydroxy-24-methylcoprostanic acid (III), in which the resulting tertiary hydroxyl group was proved to be so stable, that the dehydration procedure with glacial acetic acid and acetic anhydride that Seno (7) previously reported, failed to dehydrate this compound. Therefore, after the three secondary hydroxyl groups of methyl 3*α*, 7*α*, 12*α*, 24-tetrahydroxy-24-methylcoprostanate was protected by formylation, the uncovered tertiary hydroxyl group was treated with thionyl chloride to give the 24-chloride.

When the 24-chloride was refluxed with dimethylaniline, dehydrochlorination occurred, and the resulting oily unsaturated ester was saponified to give 3*α*, 7*α*, 12*α*-trihydroxy-24-methylcoprostenic acid (IV).

The ultra-violet spectrum of 3*α*, 7*α*, 12*α*-trihydroxy-24-methylcoprostenic acid (IV) showed the absense of *α*-*β*-unsaturated carboxylic acid grouping, and the double bond of this compound (IV) may probably exist between $C_{28}$ and $C_{24}$.

The unsaturated acid (IV) was easily hydrogenated with hydrogen and platinum oxide to yield 3*α*, 7*α*, 12*α*-trihydroxy-24-methylcoprostanic acid (V).

Melting point of methyl 3*α*, 7*α*, 12*α*-trihydroxy-24-methylcoprostanate was not depressed when admixtured with methyl trihydroxyisosterocholanate derived from the catalytic hydrogenation of methyl trihydroxyisosterocholenate obtained from the bile of *Bufo vulgaris formosus.*

Melting points of methyl 3, 7, 12-triketo-24-methylcoprostanate and its trioxime resembled the corresponding derivatives of methyl trihydroxyisosterocholanate, as shown in the following Table I.

TABLE I

*Melting Points of the Synthetic Acid, the Natural Acid and Their Derivatives*

| | Trihydroxybufosterocholanic acid | Trihydroxyisosterocholanic acid | Trihydroxy-24-methylcoprostanic acid |
|---|---|---|---|
| Acid | 200° | 200° | 192° |
| Methylester | 172° | 162° | 172° |
| Triketo acid methylester | 181—2° | 223—4° | 218—20° |
| Oxime of Triketo acid methylester | — | 232° | 230° |
| Reference | (1) | (2) | Present work |

As the results of the above-stated, it was considered that trihydroxyisosterocholanic acid may be 3*α*, 7*α*, 12*α*-trihydroxy-24-methylcoprostanic acid, but a further discussion will be deferred on the point whether there is any difference between the synthetic acid and the natural acid until the stero-bile acids are again isolated from the bile of *Bufo vulgaris formosus*, and re-examined.

## SUMMARY

1. 3α, 7α, 12α,-Trihydroxy-24-methylcoprostanic acid was synthesized from norcholylmethylketone and ethyl DL-α-bromopropionate by modified Reformatsky reaction.

2. Melting point of methyl 3α, 7α, 12α-trihydroxy-24-methylcoprostanate showed no depression, when mixed with methyl trihydroxyisosterocholanate derived from trihydroxyisosterocholenic acid (this acid was isolated from the bile of *Bufo vulgaris formosus*).

The author wishes to thank Prof. T. Kazuno for valuable suggestions during the course of this work.

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# LETTERS TO THE EDITORS

The Journal of Biochemistry, Vol. 46 No. 4, 1959

## ENZYMIC HYDROLYSIS OF A GLUTAMYL POLYPEPTIDE OF BACILLUS MEGATERIUM

Enzymic hydrolysis of a bacterial glutamyl polypeptide (GPP) produced by *B. anthracis* and *B. subtilis* has been investigated by several workers (*1–3*). In the previous paper (*4*) the author reported that GPP produced by *B. megaterium* was hydrolysed by a dog liver extract and the resulting hydrolyzate contained lower molecular peptides than the original polypeptide but no free glutamic acid. Recently it was found that there was L-glutamic acid in an enzymic digest when GPP of *B. megaterium* was incubated with the enzyme solution under a toluene layer without shaking, and glutathione was also hydrolysed liberating L-glutamic acid.

The dog liver enzyme described in this paper can be differentiated from enzymes prepared from bacterial culture by Thorne *et al.* (*1*) and Volcani *et at.* (*3*). Thus the optimum pH of the liver enzyme lies between 4.1 and 4.3, while that of the bacterial enzymes are at an alkaline pH. The liver enzyme can liberate L-glutamic acid from glutathione and cannot hydrolyse GPP of *B. anthracis.* However Volcani's enzyme can not hydrolyse glutathione and Thorne's enzyme can hydrolyse GPP of *B. anthracis.* It was considered that the present preparation is similar to Kream's preparation, but the latter was not studied in detail enzymatically. Although an enzyme which released glutamic acid from glutathione has also been reported (*5*), no detailed data about it have been published. The GPP of *B. megaterium* used contained 45 per cent of the total glutamic acid as L-glutamic acid (*6*) and is composed almost solely of $\gamma$-carboxyl linkages (*7*). As the enzyme preparation can hydrolyse not only $\gamma$-linked GPP, containing L-glutamic acid, but also glutathione, it is proposed to refer to this $\gamma$-glutamyl bond splitting enzyme as $\gamma$-glutamylase.

The enzyme solution was prepared as follows: fresh dog liver (100 g.) was homogenized in physiological saline (200 ml.) containing acetic acid (1.2 ml.). After freezing and thawing, the homogenate was centrifuged. The supernatant liquid was neutralized and the precipitate was removed. Then the solution was subjected to ammonium sulfate fractionation. The precipitate from 60 to 100 per cent saturation of ammonium sulfate was dissolved in about 10 ml. of physiological saline and dialysed against the same solution.

The action of the enzyme was studied by measuring the glutamic acid formed or by paper chromatography. Substrate solution (0.5 ml.), buffer solution (1.0 ml.) and enzyme solution (0.2 ml.) were mixed in a small tube and toluene (0.3 ml.) was added. Control batches were made by replacing substrate and enzyme with water and saline respectively. The mixture was incubated at 37°. After incubation, pH of the mixture was adjusted to about 5.0 and the mixture was heated in boiling water for 3 minutes and cooled. Precipitates were removed by centrifugation and the resulting clear solution was analysed for glutamic acid.

Experimental results are shown in the Figs. and Table. There was 100 per cent liberation of L-glutamic acid from glutathione; but from GPP of *B. megaterium* only 66 per cent of L-glutamic acid was released and no D-glutamic acid was found in the hydrolyzate. Residual L-glutamic acid and all the D-glutamic acid, therefore, seemed to consist in peptides, because neither L- or D-glutamic acid was destroyed by the enzyme under the same conditions.

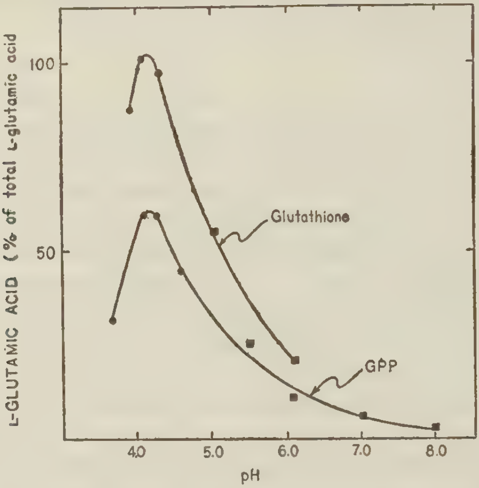

FIG. 1. Effect of pH on the hydrolysis of GPP of *B. megaterium* and glutathione. Incubation time, 22 hours at 37°. The pH was measured at the beginning of the reaction. The buffers used were $M/30$ sodium acetate-HCl (●) and phosphate (■).

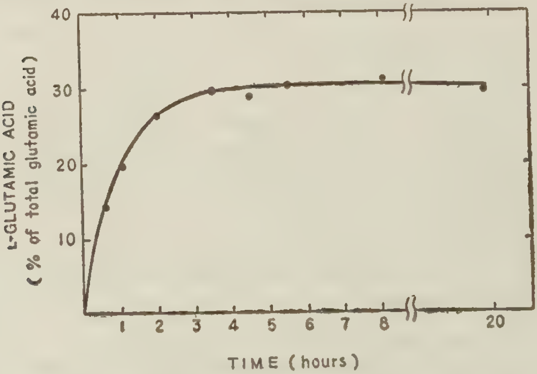

FIG. 2. Rate of release of L-glutamic acid from GPP of *B. megaterium* by a highly active enzyme preparation.

TABLE I

*Enzymic Hydrolysis of GPP and Glutathione*

| Substrate | pH | Incubation time (*hour*) | Free glutamic acid | | |
|---|---|---|---|---|---|
| | | | Total (*per cent*) | L-Isomer (*per cent*) | D-Isomer (*per cent*) |
| GPP of *B. megaterium* | 4.2 | 24 | | 30.7 | |
| » | 4.2 | 19 | | | 0 |
| » | 4.2 | 4–20 | | 30.0[1] | |
| » | 6.0 | 18 | 12.3 | 13.3 | |
| Glutathione | 4.5 | 22 | | 73 | |
| » | 4.2 | 21 | | 101 | |
| Glutamine | 4.5 | 17 | | 0 | |
| L-Glutamic acid | 4.2 | 18 | | 103 | |
| D-Glutamic acid | 4.2 | 18 | | | 120[2] |
| GPP of *B. subtilis* | 4.2 | 18 | | 22.5 | |
| GPP of *B. anthracis* | 4.2 | 19 | | 0 | 0 |

The total glutamic acid was determined by Sanger's DNP-method, L-glutamic acid by L-glutamic acid decarboxylase of *E. coli* C 6 and D-glutamic acid by D-glutamic acid oxidase of *Aspergillus ustus* f (*8*).

1) This value was obtained with a highly active enzyme preparation (see Fig. 2).

2) Paper chromatography indicated no loss of D-glutamic acid.

As this enzyme is active towards the GPP of *B. megaterium* and *B. subtilis* which are composed of both L- and D-glutamic acid, but is inactive towards that of *B. anthracis* which is composed solely of D-isomer, this enzyme seems to split the $\gamma$-L-glutamyl peptide bond. Further studies on the specificity and the purification of the enzyme and the relation to $\gamma$-glutamyl transferase are in progress.

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# ON THE HETEROGENEITY OF PROTAMINES (CLUPEINE AND SALMINE) OBTAINED FROM SPERMATOZOA OF EACH SINGLE FISH*

The heterogeneity of protamines has been reported by many investigators (*1–4*). It may possibly have resulted either on account of the maturity stages of the testis, the individual differences in the same species, or by different preparation procedures. To explain the origin of the result, each protamine, iridine and clupeine, was prepared from mature testis or the sperm of each single fish including rainbow-trout (*Salmo irideus*) or Pacific herring (*Clupea pallasii*), and was studied for its heterogeneity by means of alumina chromatography and N-terminal analysis.

In the case of iridine, the cell nuclei were isolated by Felix's plasmolysis method from a mature milt or sperm of a single rainbow-trout from a hatchery. They were then extracted with cold 0.2 $N$ hydrochloric acid, and the extract was neutralized with Amberlite IRA-400 ($HCO_3$ form) and finally lyophilized to yield a specimen of iridine. As for clupeine, each of specimens was prepared almost in a similar manner as in iridine from a frozen milt of a single herring, each obtained at three different places in Hokkaido (see Table I).

The alumina chromatography, introduced by Scanes and Tozer (*4*) for the fractionation of clupeine, was slightly modified to give a better resolution by eluting the protein from a buffered alumina column. The protamines were eluted from either one of the two alumina preparations (types A and B) by 0.45 or 0.48 $M$ phosphate buffer.

Contents of N-terminal proline in the protamines were estimated from the $R$ values (*5*) (the ratios of optical densities at 390 and 360 m$\mu$ of the UV-absorption curves in $N$ hydrochloric acid) of dinitrophenylated protamines by Sanger's method.

As shown in the elution chromatograms (Fig. 1, a and b) of several specimens of iridine and clupeine, each of these protamines prepared from a single fish, respectively, was revealed to be chromatographically heterogeneous. The heterogeneity was, regardless of the preparation procedures**, similarly found also for each protamine from a large number of the fish and was ascertained to be based on the composition difference by amino acid analysis of each chromatographic fraction.

By means of N-terminal analysis, all the specimens and fractions of iridine examined were shown to have exclusively proline in their N-termini, while the specimens of clupeine always presented proline in a molar content

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* Aided in part by a Scientific Research Grant from the Ministry of Education.

** Preparations without acid treatment also gave the same heterogeneity.

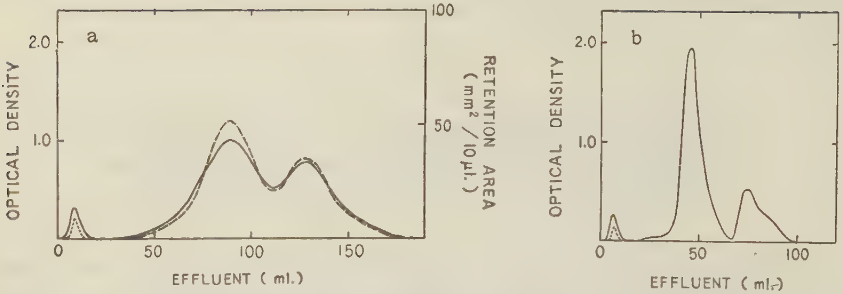

FIG. 1. Elution chromatograms of iridine (a) and clupeine (b; specimen from Okhotsk Sea, No. 5) obtained from each single fish. Iridine was eluted from a column (0.6×31 cm.) of type A alumina with 0.45 *M* $K_2HPO_4$, and clupeine from that of type B alumina with 0.48 *M* $K_2HPO_4$. Optical densities at 210 (—) and 260 mμ (···) as well as the retention method (– – –) using Ponceau 6RF were adopted for analyses (*4*).

of 0.40–0.42 in the terminus as listed on Table I, the remaining part being probably occupied by alanine (*5*). The heterogeneity of clupeine from each single fish was thus confirmed also by the N-terminal study.

In conclusion, the heterogeneity of a protamine seems to be a nature, characteristic and intrinsic to the protein, though nothing can yet be said about individual differences. Details will be reported later in this Journal.

TABLE I

*The Content of the N-Terminal Proline in Several Specimens of Clupeine from Each Single Herring Obtained at Three Differcnt Places*

| Clupeine from | Molar content of N-terminal Proline1) |
|---|---|
| Okhotsk Sea (No. 5) | 0.42 |
| Japan Sea (No. 1) | 0.40 |
| Akkeshi Bay2) (No. 1) | 0.40 |
| Akkeshi Bay2) (No. 2) | 0.42 |

1) Estimated from the *R* values of the DNP-derivatives (see the text).

2) The herring obtained in this bay is said to be a different race from the others (*6*).

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# FORMATION OF PHYCOBILIN PIGMENTS IN A BLUE-GREEN ALGA, TOLYPOTHRIX TENUIS, AS INDUCED BY ILLUMINATION WITH COLORED LIGHTS

The color changes in red and blue-green algae induced by applying colored lights to the organisms have long been recognized as the phenomenon of "chromatic adaptation" (*1*). Most of the previous studies, however, were concerned with the observation of color changes made by the naked eye, and has been little known of the quantitative aspect of the changes in the pigment components contained in algal cells. In the present study, the "chromatic adaptation" in a blue-green alga, *Tolypothrix tenuis*, was investigated through spectroscopic analysis of the organism under applying various colored lights.

A flat-bottomed flask containing algal suspension* was placed in a water thermostat regulated at 30° and lightened from its beneath with colored light obtained by the use of various color filters (see Fig. 1). To avoid heat, a glass trough filled with running tap water (5 cm, in thickness) was inserted between the filter and the light source, a 200 w. incandescent lamp. The intensity of light was measured with a Kipp thermopile and adjusted to give incident energy of $1.6 \times 10^5$ erg/sec. cm$^2$, by regulating the position of the light source. The algal suspension was continuously aerated with air containing 5 per cent $CO_2$. Aliquots of the suspension were withdrawn at appropriate intervals, and the pigment content and cellular mass (packed-cell volume) were determined. The content in phycobilin pigments was

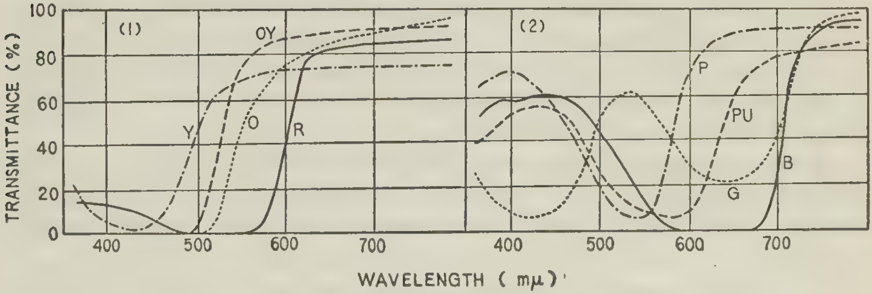

FIG. 1. Optical characteristics of the color filters. In (1), —— Red (R), ······ Orange (O), - - - Orange-yellow (OY), —-— Yellow (Y); in (2), —— Blue (B), ······ Green (G), - - - Purple (PU), —-— Pink (P).

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* The solution used had the following composition: per liter, $KNO_3$, 3.0 g.; $Na_2HPO_4 \cdot 12H_2O$, 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $CaCl_2$, 0.02 g.; $FeSO_4$, 0.004 g.; Arnon's $A_5$ solution 1 ml.

computed from spectroscopic readings made on cell-free water-extracts of algal cells as described in a previous paper (*2*). Prior to the experiments, the alga was pre-incubated for 24 to 48 hours under strong light (10,000 lux) and at a lower temperature (20°), in order to reduce the initial pigment content as much as possible (*3*).

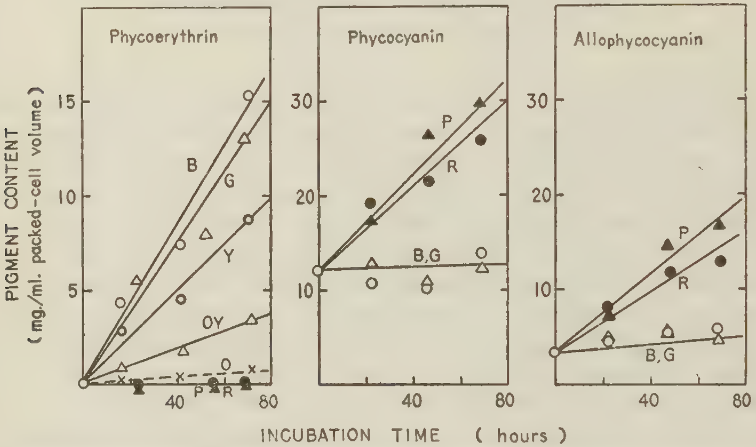

**FIG. 2.** Phycobilin formation in *Tolypothrix tenuis* under lightening various colored lights. Letters in the figures indicate the colors of lights applied; each color was produced by using the filter, the spectral characteristic of which is presented in Fig. 1.

Preliminary spectroscopic examination with suspensions of algal cells revealed that the major change observed in the chromatic adaptations of the organism consisted in alterations in the levels of phycobilin pigments, no comparable changes occurring in the levels of other colored components such as chlorophyll and carotenoids.

A marked increase in phycoerythrin content was observed on applying *blue* and *green* lights to the organism; no increase by *red* and *pink* lights (Fig. 2). The order of effectiveness in causing the increase of phycoerythrin content was:

*blue = green > yellow > orange-yellow > orange > red = pink*

The production of phycocyanin and allophycocyanin, on the other hand, was found to be strongly enhanced by *purple*, *red* and *pink* lights, but not by *blue* and *green* lights (Fig. 2). The effectiveness of various lights in this respect was in the order:

*purple = pink = red > orange > orange-yellow > yellow > blue = green*

With all phycobilin pigments, no detectable increase was observed in the dark.

These experimental results marked definitely in the fact that some photochemical process (or processes) is involved in the formation of the phycobilin pigments in the alga. The similarity in the behaviour of phycocyanin and allophycocyanin seems to suggest the occurrence of a common mechanism in the formation of these two pigments which have a close similarity in their spectral characteristics (*2*). The *blue* light, which was ineffective in causing the formation of phycocyanin and allophycocyanin was lacking in the lights of wave lengths ranging from 550 to 700 mμ, a fact indicating that the effective part of the spectrum was somewhere between these limits. Other experimental data are also consistent with this conclusion. Identical effects of *red* and *pink* lights, the former with the lights and the latter without having wave lengths shorter than 500 mμ, indicates the inefficacy of the light of shorter wave lengths.

In the case of phycoerythrin formation, the ineffectiveness of the lights having wave lengths longer than 550 mμ is definitely shown by the negative results with *orange*, *pink*, and *red* lights, is consistent with this inference. The fact that the same effectiveness in causing the phycoerythrin formation was shown by *green* light (with an energy maximum at 530 mμ and a minimum at around 400 mμ), and by *blue* light (with a broad spectrum covering the wave lengths from 400 to 550 mμ), seems to suggest that the effective spectrum acting in this case lies at wave lengths around 500 mμ (see Fig. 1).

Although our data are insufficient to allow any positive conclusion about the chemical nature of the substance responsible for the light absorption in the process under investigation, there seems to be fairly good evidence that the substance acting in the formation of phycocyanin and allophycocyanin can be neither chlorophyll nor carotenoids. In fact, the *blue* light comprising the range of wave length approximatelly corresponding to one of the absorption maxima of chlorophyll (440 mμ) was ineffective in inducing phycocyanin and allophycocyanin formation. The same line of argument is also available to the carotenoids. The possibility that one or the other of phycobilin pigments participates in the process remains open to further investigation. In the case of phycoerythrin production, on the other hand, the possibility that the phycobilin pigments are acting as the light absorbers seems to be safely excluded, since lights of wave lengths corresponding to the absorption maxima of phycoerythrin, phycocyanin and allophycocyanin (565, 620 and 650 mμ, respectively) failed to induce the pigment formation. For a similar reason, we can also exclude the functioning of chlorophyll, but a possibility remains as to the participation of carotenoids. More detailed investigations with the aim of establishing the precise action spectra of the production of each pigment component are now in progress in this laboratory.

The authors wish to extend their grateful thanks to Prof. H. Tamiya for his helpful guidance and criticism. Thanks are also due to Profs. A. Watanabe and A. Takamiya for their valuable advice.

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# CARBON DIOXIDE FIXATION INTO MALONATE IN MYCOBACTERIUM AVIUM

Enzymatic decarboxylation of malonate was studied by Hayaishi (*1*) and other investigators (*2–5*). But little information is available concerning the reverse reaction ; $CO_2$ fixation into malonate. Recently, we have found that $C^{14}O_2$ was fixed into malonate by the cell-free extract from *Mycobacteria* (*6*).

*Mycobacterium avium*, strain Takeo was grown on a glycerol-bouillon medium for 4 days. The cells were ground with sea-sand for 20 minutes in a chilled mortar, extracted with distilled water, and centrifuged at 100,000×g for 30 minutes. The supernatant fluid (extract) obtained was then incubated with 100 $\mu$ moles of $NaHCO_3$ containing $3.5\times10^5$ c.p.m., 50 $\mu$ moles of acetyl phosphate, 100 $\mu$ moles of phosphate buffer, pH 6.8, and 10 $\mu$ moles of $MgCl_2$, for one hour at 30°. The reaction was stopped by adding 6 *N* HCl, and the remaining $C^{14}O_2$ was removed by the addition of small pieces of dry ice. After centrifugation, the supernatant was adjusted to pH 9, mixed with a known amount of several organic acids as carriers and evaporated. The residue was taken up in 10 per cent $H_2SO$ , adjusted to pH less than 2, and then submitted to chromatography on Celite 535 to separate organic acids (*7*). Each eluate was titrated with 0.02 *N* NaOH and counted for $C^{14}$. The products of $C^{14}O_2$ fixation are indicated in the chromatogram of Fig. 1. Two major peaks of $C^{14}$ activity, A and C coincide with that of acetic and malonic acids respectively, and a minor peak B with succinic acid. Further identification of malonic acid was obtained by the use of automatic windowless paper chromatogram scanner as shown in Fig. 2. Such $C^{14}O_2$ fixation into malonic and acetic acids was abolished completely by heating the extract at 90° for 5 minutes or omitting acetyl phosphate from the reaction mixture described above. In addition, the reaction proceeded equally well under aerobic or anaerobic condition. Acetate replaced acetyl phosphate as a $CO_2$ acceptor only to a much lesser extent in the presence or absence of adenosine triphosphate (ATP). However, if unlabeled $NaHCO_3$ and acetyl phosphate were present together, labeled acetate was also observed to be fixed into malonate effectively as well as $C^{14}O_2$.

As shown in Table I, the coenzyme A and metal ion requirements for $C^{14}O_2$ fixation were revealed by ammonium sulfate precipitation of the extract. In contrast, ATP had inhibitory effect.

From the above results, it is suggested that the cell-free extract from *M. avium* contains an enzyme system catalyzing the reversible carboxylation of acetyl coenzyme A into malonate (or its derivative). The systems were

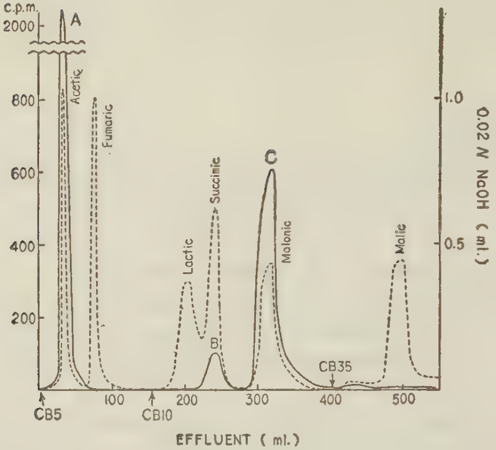

FIG. 1. Chromatogram of the products of $C^{14}O_2$ fixation with a known amount of organic acids as carriers. The system is 0.2 *N* $H_2SO_4$ on Celite 535, developed with chloroform, followed by increasing concentrations of butanol in chloroform. Solid line: $C^{14}$ activity curve. Dotted line: titration curve of added organic acids. CB5, CB10, and CB35: chloroform containing 5, 10, and 35 per cent (*v*/*v*) butanol, respectively.

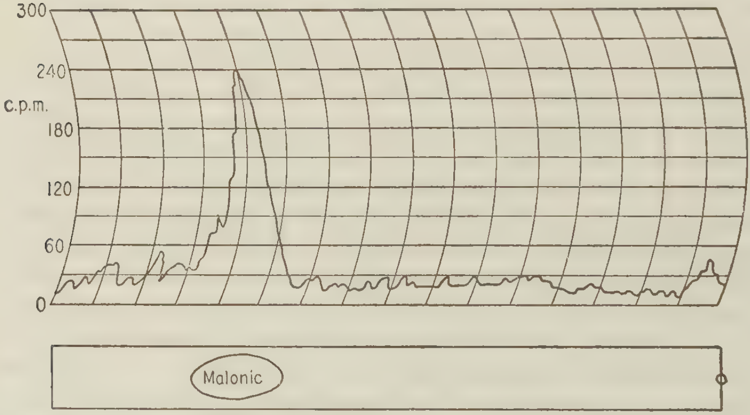

FIG. 2. Identification of malonate by automatic windowless paper chromatogram scanner. The deproteinized reaction mixture was dried up, extracted thrice with 2 ml. of ethanol, and centrifuged; the supernatant was concentrated, applied on Toyo No. 51 filter paper with malonic acid. Solvent system: butanol/formic acid/water (4 : 1 : 2). Sprayer: chlorophenol red. The radioactivity was measured by a windowless scanning device.

also found to be present in the cell-free extracts from *M. smegmatis* and *Nocardia asteroides.*

TABLE I

*Effect of Coenzyme A, ATP and Metal Ions on $C^{14}O_2$ Fixation by Ammonium Sulfate Fraction*

The precipitate between 0.25 and 0.6 saturation of ammonium sulfate from the extract was used. Other experimental conditions are the same as in the Text.

| Condition | $C^{14}O_2$ fixed into | |
|---|---|---|
| | Acetate | Malonate |
| | 0.57 μM* | 0.75 μM* |
| +CoA 200 μg. | 1.34 | 0.95 |
| +ATP 5 mg. | 0.41 | 0.39 |
| +CoA, +ATP | 1.03 | 0.47 |
| $-Mg^{++}$ | 0.02 | 0.08 |
| $-Mg^{++}$, $+Mn^{++}$ | 0.54 | 0.82 |

$$* \frac{\text{c p.m. fixed } C^{14}O_2}{\text{c.p.m. per } \mu\text{M of } NaHC^{14}O_3}$$

When this manuscript has been prepared, Wakil (*8*) reported a similar type of $CO_2$ fixation into malonate by avian liver extract independently.

The authors wish to acknowledge the helpful suggestion of Mr. Y. Okada for chromatographic separation of organic acids.

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# ENZYMATIC FORMATION OF L-GULONOLACTONE AND D-GLUCURONOLACTONE

Biosynthesis of L-ascorbic acid in animal tissues has been studied by many investigators and there are two possible explanations on its pathway. First, Burns (*1–3*) and Guha (*4–5*) demonstrated gulonolactone as an immediate precursor of ascorbic acid. Another possibility that ascorbic acid is synthesized from gulonic acid *via* 3-keto-gulonate was proposed by Lehninger, *et al.* (*6–8*).

In the previous papers (*9–11*), purification and properties of lactonase were investigated by the present writers. The absence or abnormality of gulonolactonase activity in specific manner in man, monkey, and guinea pig, all of which are unable to synthesize ascorbic acid, was demonstrated. Further, the possibility that gulonolactone was the most probable immediate precursor of ascorbic acid was shown.

Up to the present, however, the mechanism of the physiological formation of gulonolactone has not yet been clarified.

*Preparation and Assay*—Lactonase was prepared and purified from acetone-dried powder of ox liver by the method described in the preceding report (*10*) except that acetone fractionation was omitted. In order to assay lactonizing activity, hydroxlyamine hydrochloride was used as a trapping agent and hydroxamate formed from the lactone was measured colorimetrically as follows: 2 *M* Hydroxylamine hydrochloride was freshly prepared every day and the pH of its solution was adjusted to that of the reaction mixture by the addition of 4 *M* NaOH to 4 *M* $NH_2OH$ hydrochloride (otherwise the reaction mixture did not maintain its pH correctly on addition of hydroxylamine hydrochloride). Then its concentration was brought to 2 *M* by addition of water. After the reaction proceeded under the conditions described in the legend of Fig. 1, 2 ml. aliquot of the reaction mixture was mixed with 1 ml. of the reagent containing 24 per cent of conc. HCl, 6 per cent of trichloroacetic acid, and 17 per cent of $FeCl_3 \cdot 6H_2O$. Then the mixture was centrifuged 5 minutes to remove protein and, after 60 minutes' standing for the development of color, the absorbancy at 540 mμ of the clear supernatant was measured with a Beckman spectrophotometer.

The formation of hydroxamate is markedly influenced by the pH of the reaction mixture. In Fig. 2, the formation of hydroxamate derived from the lactone at various pH ranges is summarized. Amounts below 10 μM of the lactone could not be measured exactly at a pH below about 5.7.

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Following abbreviations are used: Tris, Tris-(hydroxymethyl) aminomethane; GSH, reduced glutathione; DEAE-cellulose, diethylaminoethylcellulose.

*Results and Discussion*—The observed time course of the lactone formation is shown in Fig. 1.

In order to clarify whether the lactonizing activity is the result of the

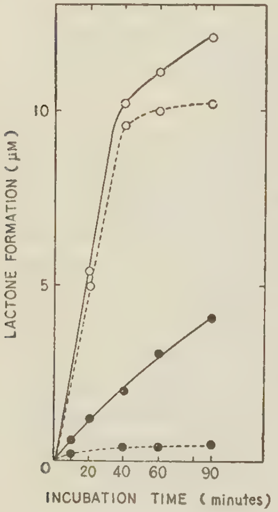


FIG. 1. Assay of lactonizing activity.

Each vessel contained: 0.05 $M$ Tris-maleate buffer (pH 6.1), 4 ml.; 0.1 $M$ $MgCl_2$, 0.2 ml.; 2 $M$ hydroxylamine (adjusted to pH 6.1), 0.6 ml.; 0.01 $M$ GSH, 0.1 ml.; enzyme, 0.5 ml.; substrate, sodium gulonate 200 $\mu$M or sodium glucuronate 120 $\mu$M; total volume, 6.0 ml. Incubation: 37°.

—○— glucuronolactone enzymatic, --○-- glucuronolactone non-enzymatic, —●— gulonolactone enzymatic, --●-- gulonolactone non-enzymatic.

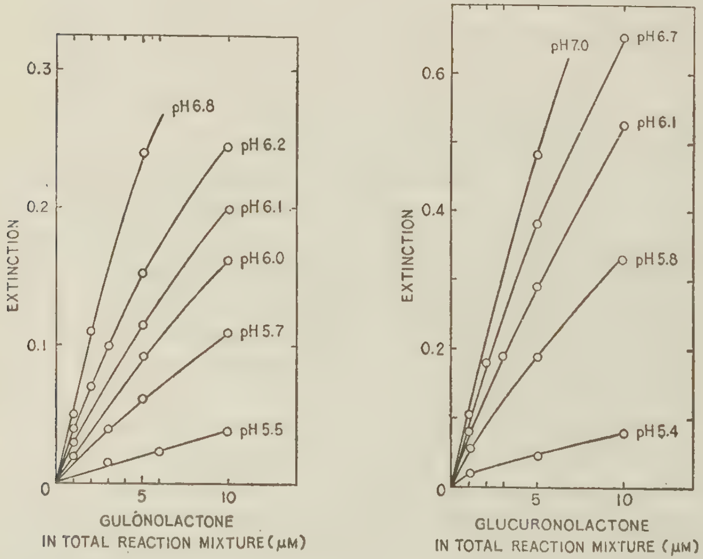


FIG. 2. Callibration and recovery1)2)3) (Effect of pH on the formation of hydroxamate derived from lactone)

1) Conditions tested were the same as in Fig. 1, but enzyme was boiled.

2) Prior to addition, the pH of hydroxylamine hydrochloride solution was adjusted to the pH of each reaction mixture.

3) Incubation: 37°, 90 minutes.

backward reaction of lactonase (lactone hydrolyzing enzyme) (*10*), some

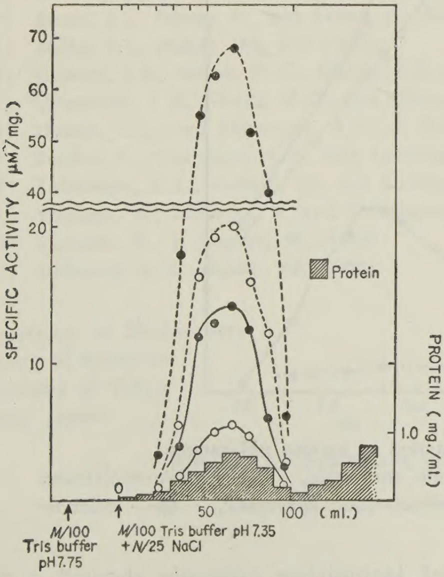
TABLE I

*Specific Activity at Each Step of Purification and Distribution of Activity in the Ammonium Sulfate Fractionation*

| | Specific activity | |
|---|---|---|
| | Lactone-hydrolyzing activity to gulonolactone* ($\mu$M/*mg.-protein*) | Lactonizing activity to gulonate ($\mu$M/*mg.-protein*) |
| Acetone powder extract | 7.0 | 1.1 |
| Heat treatment | 17.2 | 2.5 |
| Ammonium sulfate fractionation | 28.0 | 4.5 |
| DEAE-cellulose column chromatography | 81.0 | 12.2 |

| Ammonium sulfate fractionation | Distribution of total activity (%) | |
|---|---|---|
| 0–52 (%) | 13 | 12 |
| 52–67 (%) | 80 | 80 |
| 67–80 (%) | 7 | 8 |

* Lactone-hydrolyzing activity was assayed manometrically.



Lactone-hydrolyzing activity.
--●-- gulonolactone,
--○-- glucuronolactone.
Lactonizing activity.
—●— gulonate,
—○— glucuronate.

FIG. 3. Purification of enzyme with DEAE-cellulose column and specific activity of each eluate.

comparative investigations were carried out as follows: The increase in the rate of specific activity in each step of purification and the distribution pattern of activity in the step of ammonium sulfate fractionation are shown in Table I. Similar proportions were noticed in both lactone-hydrolyzing and lactonizing activities. Furthermore, as can be seen in Fig. 3, each eluate from DEAE-cellulose column also gave a similar pattern in respect of lactone-hydrolyzing and lactonizing activities, and also the activity ratio between glucuronate and gulonate remained constant at about 1 : 2.5.

TABLE II

*Inhibition and Its Reversal*

| | PCMB | $Hg^{++}$ |
|---|---|---|
| Concentration (*M*) | $2\times10^{-6}$ | $1\times10^{-6}$ |
| $Mg^{++}$(+) | 100 | 100 |
| Inhibition (%) $Mg^{++}$ (−) | 100 | 100 |
| Concentration (*M*) | $1.6\times10^{-4}$ | $3.2\times10^{-4}$ |
| GSH $Mg^{++}$(+) | 100 | 50 |
| Reversal (%) $Mg^{++}$ (−) | 0 | 50 |

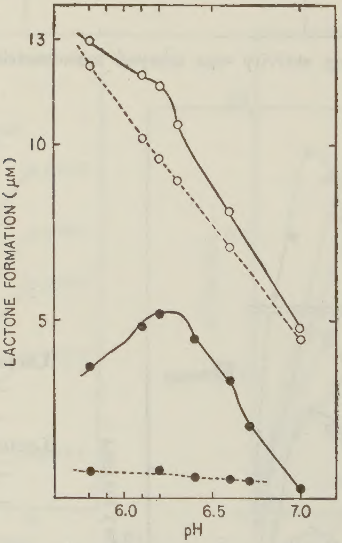


FIG. 4. Lactone formation at various pH ranges.
—○— glucuronolactone enzymatic, --○-- glucuronolactone non-enzymatic, —●— gulonolactone enzymatic, --●-- gulonolactone non-enzymatic.

Moreover, the properties of lactonizing principle showed a striking resemblance with those of the lactonase (*10*), as follows: The lactonizing activity was inhibited by heavy metal ions and SH-blocking agents. Reversal of these inhibition by GSH was observed and this process depended on the presence of $Mg^{++}$ ions (Table II). Lactonizing activity was stimulated about 3-fold by $10^{-2}\,M$ $Mg^{++}$ or $10^{-5}\,M$ $Mn^{++}$ ions.

The above findings suggest that a single enzyme is responsible for lactonizing and lactone-hydrolyzing activities, that is, lactonizing activity is due to the backward reaction of lactonase.

The optimum pH of lactonizing activity is 6.1–6.3 with regard to both gulonate and glucuronate, in Tris-maleate buffer, whereas the optimum pH of lactone-hydrolyzing activity is 6.8–7.0 in the bicarbonate buffer as shown in the preceding report (*10*). This difference of optimum pH might be due to the fact that hydrolysis of lactone is a reaction of acid formation, which occurs in general more easily under alkaline conditions (Fig. 4).

Under the conditions shown in Figs. 1 and 4, 5–15 per cent of the glucuronate added can be lactonized, the majority of which is lactonized non-enzymatically. On the other hand, 2–4 per cent of the gulonate added can be lactonized only enzymatically, but not non-enzymatically. Therefore, it seems that the significance of this enzyme on the lactonization is to lactonize gulonate, glucuronolactone being formed non-enzymatically.

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